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(54) Title: SECRETED HUMAN FAS ANTIGEN

(57) Abstract

Disclosed is a natural, soluble form of the Fas antigen polypeptide that is secreted by human cells and is the result of alternative mRNA processing in the cell. This variant of Fas is shown to be present at higher levels in patients with SLE and AILD. Also disclosed are nucleic acids encoding this unique form of the Fas antigen and methods for making and using the protein and nucleic acids, including various diagnostic embodiments such as ELISAs.

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DESCRIPTION

SECRETED HUMAN FAS ANTIGEN

BACKGROUND OF THE INVENTION

The U.S. Government owns rights in the present invention pursuant to grant numbers P60 AR20614, P50 AI23694, P01 AR03555 and R01 AI30744 from the National Institutes of Health.

1. Field of the Invention

The present invention relates generally to the fields of diagnostic tests and immunology. More particularly, it concerns the discovery of a natural soluble form of the Fas antigen. The invention describes novel Fas antigen variants, and the nucleic acid sequences encoding such variants, that may be used, *e.g.*, in diagnostic ELISAs or nucleic acid hybridization embodiments.

2. Description of the Related Art

Programmed cell death or apoptosis is a fundamental mechanism in the development of an organism, and occurs from embryogenesis throughout life (Vaux *et al.*, 1993). Much remains to be learned about the apoptotic process at the molecular level, however the more classical type of programmed cell death is thought to require activation of a set of genes that lead to DNA fragmentation and subsequent apoptotic morphological changes.

A membrane receptor-like protein, Fas, has been reported to be involved in this process. Fas antigen is a membrane-associated polypeptide that consists of 306 amino acids in mouse (Watanabe-Fukunaga *et al.*, 1992a)

and 335 amino acids in human (Itoh *et al.*, 1991; Oehm *et al.*, 1992). The human Fas antigen has a signal peptide of 16 amino acid residues at its amino terminus, an extracellular domain of 157 amino acid residues, a hydrophobic transmembrane domain of 17 amino acid residues and a cytoplasmic tail of 146 amino acids at its carboxyl terminus. The extracellular domain can be divided into three cysteine-rich subdomains. The cytoplasmic region contains the domain that is required for initiation of the apoptotic response (Itoh and Nagata, 1993).

Structural homology places Fas in the superfamily including tumor necrosis factor receptors, nerve growth factor receptor and CD40 (Watanabe-Fukunaga *et al.*, 1992a; Itoh *et al.*, 1991; Oehm *et al.*, 1992; Itoh and Nagata, 1993). In the immune system, the occurrence of massive cell death in the thymus is at least partly due to an apoptotic process mediated through signaling by the Fas antigen (Yonehara *et al.*, 1989; Trauth *et al.*, 1989) that is also involved in T cell-mediated cytotoxicity (Rouvier *et al.*, 1993). Abnormally expressed Fas is observed in the *lpr* mutant mouse strains (Watanabe-Fukunaga *et al.*, 1992b; Adachi *et al.*, 1993; Wu *et al.*, 1993) that display lymphoproliferative disorders (Frizzera *et al.*, 1989; Saito *et al.*, 1992).

Surface expression of the Fas antigen has been described in various cell types, including activated T- and B-lymphocytes (Itoh *et al.*, 1991). However, a soluble form of the Fas antigen has not been described. Soluble forms of various receptors including cytokine receptors and hormone receptors, and of other membrane molecules including major histocompatibility complex class I and CD60 molecules, have been previously characterized (Fernandez-Botran, 1991). Soluble receptors may be produced through proteolytic cleavage of

membrane-bound receptors as is the case for IL-2 receptor (Josimovic-Alasevic, 1988) and human tumor necrosis factor (TNF) receptor (Schall *et al.*, 1990). They may also be produced as translation products of alternatively spliced mRNA as is the case for murine IL-4 receptor (Mosley *et al.*, 1989)) and murine and human IL-7 receptor (Goodwin *et al.*, 1990).

The secreted receptors represent truncated forms of the membrane-bound receptors and therefore lack the transmembrane domains but retain the ability to bind ligand with a similar affinity to that of their membrane-bound counterparts. As a result, these soluble receptors constitute effective inhibitors and play an important role in the regulation of the activity of the membrane bound receptors.

A synthetic extracellular domain of Fas having 157 amino acids has been proposed as a useful agent (EP 0,510,691). However, this form of "soluble Fas" is obtained from a man-made construct and is not believed to reflect the physiological situation. The identification and isolation of a soluble Fas protein that truly exists in nature would represent a significant development. Such a discovery would potentially offer new diagnostic, or even treatment methods, for lymphoproliferative and autoimmune disorders.

SUMMARY OF THE INVENTION

The present invention, in a general and overall sense, arises from the surprising discovery of a natural, soluble form of the Fas antigen. This form of the antigen is a component of human cells, and arises naturally from alternative mRNA processing in the cell. The novel protein is a truncated Fas variant that lacks a stretch of polypeptide to anchor it in the cell membrane.

This allows the unique intracellular-extracellular Fas domain mutant protein to be secreted from the cell.

Another even more surprising discovery of the invention is that naturally occurring soluble Fas is present at higher levels in the sera of patients with autoimmune diseases, such as systemic lupus erythematosus (SLE). Earlier work analyzing only the mRNA had suggested that there was no difference in splice variants comparing normal controls to patients with SLE or AILD. Moreover, the present inventors unexpectedly found the serum levels of soluble Fas protein to correlate with autoimmune disease (Example IX, FIG. 6).

A particularly novel feature of the soluble Fas of this invention is that it is a component of both human cells and is found within human sera. The protein is also unique structurally, as it combines both elements of the intracellular Fas domain and the extracellular Fas domain and yet does not contain a stretch of polypeptide sufficient to anchor it within the plasma membrane. These features, amongst others, serve to distinguish the proteins and polypeptides of the present invention from other man-made Fas variants described in the prior art, including the synthetic extracellular Fas domain of 157 amino acids described by Nagata *et al.*, (EP 0,510,691). The Fas proteins of the invention may variously be referred to as soluble Fas, truncated Fas, a Fas variant or a mutant Fas.

Fas Proteins, Polypeptides and Peptides

It will be appreciated that the invention is not limited to Fas proteins purified from human or other mammalian cells, and also includes so-called "recombinant" proteins. Unique nucleic acid segments were discovered to be present within human cells that

encoded the soluble and secreted form of Fas. As the novel nucleic acid sequences are disclosed herein, it is evident they may be used to prepare large quantities of Fas proteins, polypeptides and peptides. It is not the question of recombinant *vs.* native that is important in defining the Fas proteins of the invention, but the combination of both the internal and external domains with no substantially hydrophobic linking portion.

Accordingly, in certain embodiments, the invention provides compositions that comprise an isolated Fas antigen protein, polypeptide or peptide that has a portion of the Fas antigen intracellular region, a portion of the Fas antigen extracellular region, and wherein at least a portion of the Fas antigen transmembrane region has been deleted or otherwise removed. The Fas antigens will preferably include sequences in accordance with the human Fas antigen polypeptide. By "a portion of the Fas antigen transmembrane region" is meant a functional portion of the known section that normally resides in the membrane to anchor the full-length Fas protein receptor at the cell surface.

The human Fas transmembrane region is known in the art to essentially have the following sequence: Leu-Gly-Trp-Leu-Cys-Leu-Leu-Leu-Leu-Pro-Ile-Pro-Leu-Ile-Val-Trp-Val (position 174 to position 190 of SEQ ID NO:2). Fas proteins and peptides in accordance with the invention will thus lack a portion of this sequence sufficient to render the protein secretable by the cell. It is not necessary for the entire 17 amino acid stretch to be deleted, just a sufficient number of these residues to allow passage of the resultant protein or peptide through a lipid membrane. This property may be readily determined, *e.g.*, by protein vesicle transport studies or by inserting a recombinant vector encoding a Fas variant

into a recombinant host cell, such as a COS cell, and determining whether the Fas protein appears in the culture supernatant, *e.g.*, using antibody detection.

As a particular Fas protein of the invention, namely FasΔ1, does not itself lack the complete transmembrane region but is still secreted by cells, both in culture and within the human body, this demonstrates that the complete region does not have to be removed to allow secretion. Preferred Fas proteins will generally be those that lack a significant portion of the Fas antigen transmembrane region, such as lacking 11, 12, 13, 14 or more residues from this region, and that are still secretable. The presence of a small number of hydrophobic amino acids between the intracellular and extracellular domains is not contemplated to prevent secretion.

It will be understood that the Fas proteins and peptides of the invention do not have to include essentially full-length intracellular or extracellular Fas regions or domains. Indeed, the length of the proteins and peptides is virtually limitless so long as at least a portion of each domain is present and so long as the domains are not linked by a significant hydrophobic region. The chosen length will naturally depend on the desired function of the resultant Fas construct.

Short peptides having unique contiguous sequences may be desired for use in generating polyclonal or monoclonal antibodies directed against the primary sequence of the new domain junction point of the Fas protein. Such peptides may be conjugated to a carrier protein for immunization purposes if necessary. Longer polypeptides and proteins may also be used to generate polyclonal or monoclonal antibodies against Fas, and to

select for antibodies that bind to the novel structural configuration that will be apparent at the new domain junction area of the Fas protein.

Fas proteins and peptides in accordance with the invention are not required to have an approximately equal portion of the intracellular or extracellular Fas regions. Proteins including a substantial portion of the extracellular Fas region may be employed in isolating and purifying Fas ligands, *e.g.*, from sera. For such purposes, the presence of a significant portion of the intracellular region is not required. Compounds having the extracellular Fas region may be linked to a solid support to form an affinity column for purification purposes.

The compositions that include the isolated Fas antigen proteins or polypeptides of the invention are themselves virtually limitless so long as the Fas is in isolated form, *i.e.* somewhat purified. "Compositions" may thus be aqueous solutions of isolated Fas, *e.g.*, Fas in pharmaceutically acceptable buffers, or suitably aliquoted in ELISA buffers (*e.g.*, in combination with BSA or Tween-20® or such like), or culture supernatants containing Fas secreted from a recombinant host cell, or even a significantly purified sample of Fas isolated from human sera. A composition in this sense may also be a lyophilized form of Fas, or a solid support, such as a column or an ELISA plate, to which Fas is attached.

Fas proteins, polypeptides or peptides may also be conjugated to other proteins or peptides to make a fusion, hybrid or chimeric protein. The other protein portions may be designed to act as inert carriers, or for use as an antigenic tag, or for means of ready purification on an affinity column, as is well known in the art. Carriers are particularly contemplated for use

with peptides for use in immunization embodiments. Suitable carriers include albumins, such as BSA and ovalbumin, and other proteins such as keyhole limpet hemocyanin (KLH). Other engineered proteins contemplated for use include, *e.g.*, glutathione-S-transferase (GST), ubiquitin, ß-galactosidase and the like.

The terms "isolated" and "purified" as used herein, are intended to refer to a soluble Fas composition, isolatable from human cells, human sera, or recombinant host cells, wherein the Fas is purified to any degree relative to its naturally-obtainable state, *e.g.*, in this case, relative to its purity within a human serum sample. Purified Fas therefore also refers to transmembrane-deficient Fas, free from the environment in which it may naturally occur, *e.g.*, in intact cells or in sera or other body fluids.

Generally, "isolated" refers to a Fas composition which has been subjected to fractionation to remove various non-Fas components, and which composition substantially retains its structural integrity and activity. Where the term "substantially purified" is used, this will refer to a composition in which Fas forms the major component of the composition, such as constituting about 50% of the proteins or peptides in the composition or more.

Various methods for quantifying the degree of purification of Fas will be known to those of skill in the art in light of the present disclosure. These include, *e.g.*, determining the activity of an active fraction, assessing the number of polypeptides within a fraction, *e.g.*, by SDS/PAGE analysis, or by assessing the immunoreactivity in comparison to the total protein content.

Techniques suitable for use in protein purification are well known to those of skill in the art. These include, *e.g.*, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. Such methods may thus be employed to purify the Fas proteins of the invention. A preferred purification method will likely include an affinity purification step of some sort that uses anti-Fas antibodies. Further preferred methods are those that use recombinant cells as a starting point, where it is likely that merely collecting the Fas-containing material from the culture supernatant will represent a significant purification step.

Although preferred for use in certain embodiments, there is no general requirement that the Fas compositions of the invention always be provided in their most purified state. Indeed, it is contemplated that less substantially purified Fas compositions will have utility in certain embodiments. These include, *e.g.*, the coating of an ELISA well in which any non-specific binding sites will subsequently be blocked, and the generation of an immune response, in which anti-Fas specific antibodies will later be selected away from other antibodies.

The Fas proteins, polypeptides and peptides of the invention will preferably include a sequence from around the breakpoint of the prominent form of soluble Fas discovered by the inventors, termed FasΔ1. The peptides will thus be at least about 10 residues long and have about a 10 amino acid long contiguous sequence as set forth by the contiguous amino acid sequence from Lys at position 164 to Glu at position 173 of SEQ ID NO:4. More

preferably, they will include the human soluble Fas antigen contiguous amino acid sequence Lys Cys Lys Glu Glu Val Lys Arg Lys Glu (position 164 to Glu at position 174 of SEQ ID NO:4). These are the 10 amino acids that span the breakpoint discovered in natural soluble Fas secreted by human cells.

The term "span the breakpoint" is used herein to refer to both contiguous nucleic acid and contiguous amino acid sequences that have one sequence portion derived from the Fas intracellular domain and one sequence portion derived form the Fas extracellular domain, and which do not contain a significant sequence portion derived from the Fas transmembrane domain. Thus, "breakpoint" in the present context does not refer to the breakage of chromosomal material and translocation to another chromosome. As the soluble Fas proteins described herein are newly discovered, proteins and peptides with contiguous sequences spanning the breakpoint have not been previously described or engineered by man.

Peptide sequences that span the breakpoint do not need to be equidistant around the breakpoint, they can thus be "offset" and include more sequence elements from one Fas domain than the other. Ser Asn Thr Lys Cys Lys Glu Glu Val Lys Arg (position 161 to 171 of SEQ ID NO:4), and Lys Glu Glu Val Lys Arg Lys Glu Val Gln Lys (position 166 to 176 of SEQ ID NO:4), are exemplary peptide sequences to illustrate this point. A Fas antigen polypeptide that includes about a 20 residue long contiguous breakpoint-spanning sequence from SEQ ID NO:4 may, for example, include 15 amino acids from the intracellular domain and five from the extracellular domain, or *vice versa*, so long as it includes the sequence Lys Cys Lys Glu Glu Val Lys Arg Lys Glu. Such offset peptides may also be considerably longer and may

include about equal sequence elements from each domain, or may be predominantly composed of sequence elements from one or other of the domains, so long as they include breakpoint-spanning element.

Fas proteins and peptides may also include a sequence region that consists of at least about a 25 or 30 amino acid long contiguous sequence as set forth by any contiguous sequence from SEQ ID NO:4, so long as it includes the breakpoint sequence or an equivalent thereof. Proteins having larger contiguous sequence regions are also contemplated, *e.g.*, those having about 35, 40, 50, 60, 70, 80, 90, 100, 120, or about 150, 200, 250 or about 300 contiguous amino acids in accordance with a contiguous sequence from SEQ ID NO:4. "About" in about 20, about 30, *etc.*, means a range of from 1 to 4 amino acids longer or shorter than the stated length, with it being understood that 9 or 10 or so is still the minimum length contemplated.

A Fas construct comprising at least a 298 residue long contiguous sequence as set forth by the contiguous amino acid sequence from Arg at position 17 to Val at position 314 of SEQ ID NO:4 is one preferred composition of the invention. Other preferred Fas proteins are those comprising a sequence region having the 314 residue long contiguous amino acid sequence of SEQ ID NO:4, *i.e.* also including the leader peptide.

As mentioned, the proteins are not limited in their maximum length and can thus be longer than 298 or 314 amino acids. They may be about 400, 500, 600 or 700 amino acid residues or longer and may include substantial portions of non-Fas sequences if desired.

The soluble Fas proteins and polypeptides of the invention have many uses. One important utility is as

the standard-curve component of an ELISA to detect soluble Fas in samples, such as human serum samples. The formation of a standard curve is desired to be able to easily "read off" the amount of Fas within a sample from an O.D. value of a given ELISA well. Anti-Fas ELISAs have definite utility in the detection of Fas *per se*. They also have an important diagnostic utility as the present inventors have discovered that human subjects with the autoimmune disease SLE have significantly elevated levels of soluble Fas in their sera.

Further possible uses of the proteins and peptides include their use as immunogens to generate specific polyclonal or monoclonal antibodies. Such antibodies may be used, *e.g.*, in diagnostic ELISAs as described above, or even *in vivo* to eliminate excess soluble Fas as a means to increase apoptosis in autoimmune diseases, malignancies and aging. Even further potential uses of Fas proteins and peptides include their use in prolonging the survival and proliferation of T cells in culture after T cell stimulation; their use in purifying the Fas ligand by, *e.g.*, contacting a sample believed to contain the ligand, such as sera, under conditions effective to promote binding and then obtaining the substance thus bound; their use in expression cloning embodiments as specific probes to screen cDNA libraries to identify and clone the Fas-ligand; and their use in binding to anti-human Fas antibodies to neutralize such antibodies, and consequently, to inhibit antibody-mediated apoptosis. In the latter context, increasing soluble Fas may be used to decrease apoptosis *in vivo* and decrease cell death in stroke or myocardial infarction.

However, the utility of the Fas proteins and peptides does not have to be a novel utility and does not have to relate to apoptosis in general. As such, the proteins and peptides may be employed as standards in

protein assays, as molecular weight markers on SDS gels, and the like.

In further embodiments, the present invention provides antibodies, including polyclonals and monoclonals, which antibodies are capable of binding to a Fas antigen epitope that includes a portion of the Fas antigen intracellular region, a portion of the Fas antigen extracellular region and that does not include a significant portion of the Fas antigen transmembrane region. Such anti-Fas antibodies, and labeled conjugates thereof, will thus bind to unique epitopic sites only available on the type of Fas proteins and peptides created by alternative splicing or synthetic versions of such proteins and peptides. The antibodies may also be used in the diagnostic ELISA disclosed herein, as well as in other immunoassays, such as Western blots, RIAs, dot blots, *in situ* hybridization and the like; in Fas protein purification; and may even be administered to an animal as part of a treatment regimen for an autoimmune disease.

The novel antibodies will bind to a sequential linear epitope or to as assembled topographic site epitope. The linear epitopes will generally be those that include contiguous intracellular and extracellular domain sequence elements, as exemplified by Lys Cys Lys Glu Glu Val Lys Arg Lys Glu (position 164 to 173 of SEQ ID NO:4), or slightly off-set peptide sequences, such as Lys Glu Glu Val Lys Arg Lys Glu (position 166 to 173 of SEQ ID NO:4) or Lys Cys Lys Glu Glu Val Lys Arg (position 164 to 171 of SEQ ID NO:4), or any similar peptide of between about 8 and about 20 amino acids in length that includes the contiguous breakpoint sequence Lys Cys Lys Glu Glu Val Lys Arg (position 164 to 171 of SEQ ID NO:4).

Nucl ic Acid Segments and V ctors

This invention also provides novel nucleic acid segments, probes, primers and vectors for use in molecular biological embodiments, including diagnostic and screening assays and protein expression. The inventors discovered, through the use of the reverse-transcription (RT)-polymerase chain reaction (PCR™), and through subsequent cloning and sequencing of the amplified products, that human PBMCs naturally contain *fas* mRNA variants lacking certain nucleotide regions that encode the transmembrane domain of the Fas antigen. They further discovered that these transcripts represent a substantial proportion of total *fas* mRNA species in the cells, as revealed by RNase protection analysis, and that the encoded protein products are present in the cytoplasm as a soluble form and are also secreted from the cells.

In the terminology employed in the present application, the nucleotide sequence of the full-length *fas* from position 170 to position 1336 is SEQ ID NO:1 (1167 base pairs), and the corresponding deduced amino acid sequence is SEQ ID NO:2 (335 amino acids, including the 319 of the soluble protein and the 16 residue leader peptide). The nucleotide sequences that encode the novel soluble Fas proteins are exemplified by SEQ ID NO:3, which is a particularly preferred sequence, and also SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9. The corresponding deduced amino acid sequence of the preferred Fas deletion mutant, FasΔ1, is SEQ ID NO:4, which is a 314 amino acid sequence that includes the 298 residues of the soluble protein and a 16 residue leader peptide. Other soluble Fas variants described herein are those with sequences of SEQ ID NO:6, SEQ ID NO:8, and SEQ ID NO:10.

Important aspects of the present invention thus concern isolated DNA segments and recombinant vectors that encode a mutant Fas antigen protein, polypeptide or

peptide that includes a portion of the Fas antigen intracellular region, a portion of the Fas antigen extracellular region, and that lacks a portion, or a significant portion, of the Fas antigen transmembrane region. Also provided, through the application of DNA technology, is the creation and use of recombinant host cells that express the mutant Fas proteins, polypeptides or peptides. The DNA segments are isolatable from mammalian cells, such as PBMCs or purified T cells, and are preferably obtained from human cells.

As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding a mutant or soluble Fas product refers to a DNA segment that contains such *fas* coding sequences yet is isolated away from, or purified free from, total genomic DNA, *e.g.*, total DNA of the T cell from which the DNA segment is obtained. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

Similarly, a DNA segment comprising an isolated or purified mutant or soluble *fas* gene refers to a DNA segment including Fas encoding sequences and, in certain aspects, regulatory sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences, cDNA sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides or peptides.

"Isolated substantially away from other coding sequences" means that the gene of interest, in this case the transmembrane-defective *fas* gene, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode a Fas protein or peptide that includes within its amino acid sequence at least a 10 amino acid long contiguous sequence as set forth by the contiguous amino acid sequence from Lys at position 164 to Glu at position 173 of SEQ ID NO:4. DNA segments encoding any of the previously described proteins or peptides that include longer contiguous sequences are also provided, including DNA species that encode the full-length protein termed FasΔ1 of 298 amino acids (or its 314 unprocessed counterpart).

The term "a sequence as set forth in SEQ ID NO:4" means that the sequence substantially corresponds to a particular contiguous portion of SEQ ID NO:4, usually referring to the breakpoint spanning portion, and has relatively few amino acids that are not identical to, or a biologically functional equivalent of, the amino acids of the SEQ ID NO:4 breakpoint region. The term "biologically functional equivalent" is well understood in the art and is further defined in detail hereinbelow. Accordingly, the 10 or 20 amino acid long contiguous sequences from the breakpoint spanning region of SEQ ID NO:4 are not required to have exactly the same amino acid

at every single point on the sequence. Peptides with one, two, three or so different amino acids are contemplated to be functional equivalents, so long as no more than two or so of the exchanged residues are hydrophobic amino acids.

Where the contiguous portions from SEQ ID NO:4 referred to are more distant from the extracellular-intracellular junction, a considerably larger amount of variation is tolerated. Therefore, DNA sequences that have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:4 will be sequences that are "as set forth in SEQ ID NO:4" for purposes other than the immediate breakpoint sequence.

In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that include within their sequence a contiguous nucleic acid sequence as set forth by a contiguous nucleic acid sequence from the region of SEQ ID NO:3 that spans the breakpoint, including sequences that are not equidistant around the breakpoint. By way of example only, sequences may start at about position 524, 522, 520 or 515 of SEQ ID NO:3 and may extend until about position 537, 539, 541 or 544 of SEQ ID NO:3. Of course, longer sequences are also envisioned, *e.g.*, those starting from about position 500 and extending to about position 570; up to and including the full-length sequence of SEQ ID NO:3.

The term "as set forth in SEQ ID NO:3" is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to the defined portion of SEQ ID NO:3 and has relatively few codons that are not identical, or functionally

equivalent, to the codons of SEQ ID NO:3. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid (see the codon table included herein), such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids.

It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5′ or 3′ sequences, and yet still be as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including having substantially the sequence elements as those of the breakpoint region. The addition of terminal sequences particularly applies to nucleic acid sequences that may, *e.g.*, include various non-coding sequences flanking either of the 5′ or 3′ portions of the coding region or may include various internal sequences, *i.e.* introns, which are known to occur within genes.

Excepting flanking regions, and allowing for the degeneracy of the genetic code, sequences that have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of nucleotides that are identical to the nucleotides of SEQ ID NO:3 will be sequences that are "as set forth in SEQ ID NO:3". Sequences that are effectively the same as those set forth in SEQ ID NO:3 may also be functionally defined as sequences that are capable of hybridizing to a nucleic acid segment containing the complement of SEQ ID NO:3 under relatively stringent conditions. Suitable relatively stringent hybridization conditions will be well known to those of skill in the art and are clearly set forth herein in the hybridization embodiments of the detailed examples.

Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:3. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementarity rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of SEQ ID NO:3 under relatively stringent conditions such as those described herein.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared that include a certain contiguous stretch identical to, or complementary to, a SEQ ID NO:3 breakpoint region, but that are up to about 10,000 or about 5,000 base pairs in length, with segments of about 3,000 being preferred in certain cases. DNA segments with total lengths of about 1,000, about 500, about 200, about 100 and about 50 base pairs in length (including all intermediate lengths) are also contemplated to be useful.

It will be readily understood that "intermediate lengths", in the context of both proteins and nucleic

acids, means any length between the quoted ranges, such as 14, 15, 16, 17, 18, 19, 20, *etc.*; 21, 22, 23, etc; 30, 31, 32, *etc.*; 50, 51, 52, 53, *etc.*; 100, 101, 102, 103, *etc.*; 150, 151, 152, 153, *etc.*; including, for nucleic acids, all integers through the 200-500; 500-1,000; 1,000-2,000; 2,000-3,000; 3,000-5,000; 5,000-10,000 ranges, up to and including sequences of about 12,001, 12,002, 13,001, 13,002 and the like.

It will also be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NO:3 and SEQ ID NO:4. Recombinant vectors and isolated DNA segments may therefore variously include the Fas coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region or they may encode larger polypeptides that nevertheless include Fas-coding regions.

In regard to functionally equivalent proteins or peptides, such may be created *via* the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein or to test Fas mutants in order to further examine their activity at the molecular level.

As mentioned above, one may also prepare fusion proteins and peptides where the Fas coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (*e.g.*, proteins that may be purified by affinity chromatography

and enzyme label coding regions, respectively). This is readily achieved by the standard DNA manipulation techniques well known to those of skill in the art (see *e.g.*, Sambrook *et al.*, 1989 incorporated herein by reference).

Recombinant vectors form important further aspects of the present invention. Particularly useful vectors are contemplated to be those vectors in which the coding portion of the DNA segment, whether encoding a full-length protein or smaller breakpoint-centered peptide, is positioned under the control of a promoter. The promoter may be in the form of the promoter that is naturally associated with a *fas* gene(s), *e.g.*, in T cells, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein.

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with a *fas* gene in its natural environment. Such promoters may include mammalian promoters normally associated with other genes, and/or promoters isolated from a bacterial, viral or other eukaryotic cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.*, (1989). The promoters employed may be constitutive, or inducible, and

can be us d under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. A currently preferred promoter is the CMV promoter/enhancer element.

In connection with expression embodiments to prepare recombinant Fas proteins and peptides, it is contemplated that DNA segments encoding the entire soluble Fas protein may be used, as may shorter DNA segments where needed to direct the expression of Fas peptides, or epitopic core regions, such as may be used to generate anti-Fas antibodies. In the nucleic acid vectors, the Fas antigen coding region may be positioned in a reverse orientation to that of the promoter, so that the promoter directs the expression of an antisense nucleic acid segment. Such antisense constructs are useful in hybridization.

Recombinant host cells that incorporate a nucleic acid segment, such as vector, that encodes a soluble Fas compound are another aspect of the invention. The cells may be prokaryotic host cells, such as *Escherichia coli*; eukaryotic microbes, such as yeast cultures; or cells derived from the propagation of vertebrate cells in culture, *e.g.*, VERO and HeLa cells, Chinese Hamster Ovary (CHO) cells and, preferably, COS cells.

This provides for further methods of the invention that include using the DNA segments disclosed herein. For example, to use a DNA segment in terms of recombinant expression, one would, generally, prepare a recombinant vector in which a soluble Fas antigen coding region is positioned under the control of a promoter, and introduce the vector into a recombinant host cell. One would then culture the recombinant host cell under conditions effective to allow expression of the mutant Fas antigen polypeptide, enabling the expressed, and possibly,

secreted, soluble Fas antigen polypeptide to be collected. The expressed Fas antigen polypeptide may be purified by isolating away from total recombinant host cell components.

The soluble *fas* gene and DNA segments may even be used in connection with somatic expression in an animal or in the creation of a transgenic animal. In such embodiments, the use of a recombinant vector that directs the expression of the complete soluble Fas protein would be preferred.

In addition to their use in directing the expression of Fas proteins, the nucleic acid sequences disclosed herein also have a variety of other uses. For example, they also have utility as probes or primers in nucleic acid hybridization embodiments. As such, it is contemplated that nucleic acid segments that comprise a sequence region that consists of at least a 10 to 14 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 10 to 14 nucleotide long contiguous sequence around the breakpoint of SEQ ID NO:3 will find particular utility. Longer contiguous identical or complementary sequences, *e.g.*, those of about 20, 30, 40, 50, 100, 200, 500, 1000 (including all intermediate lengths) and even up to full-length sequences of about 1336 nucleotides in length, will also be of use in certain embodiments.

The ability of such nucleic acid probes to specifically hybridize to soluble Fas-encoding sequences will enable them to be of use in detecting the presence of complementary sequences in a given sample. Also, the area immediately surrounding the structural gene for Fas antigen variants may be characterized to identify regulatory regions and other genes related to the regulation, production, and processing of the Fas variant

polypeptide. Further uses envisioned include the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Nucleic acid molecules having sequence regions consisting of contiguous nucleotide stretches of about 10 to 14, about 15 to 20, 30, 50, or even of about 100-200 nucleotides or so, identical or complementary to SEQ ID NO:3 are particularly contemplated as hybridization probes for use in, *e.g.*, Southern and Northern blotting. The total size of fragment, as well as the size of the complementary stretch(es), will ultimately depend on the intended use or application of the particular nucleic acid segment.

Diagnostic and other Methods

The invention also includes various methods for diagnosing Fas-associated diseases, which methods generally involve determining the amount of a mutant Fas antigen lacking a transmembrane region present within a biological sample from a patient suspected of having a Fas-associated disease. The finding of an increased amount of such a soluble or mutant Fas antigen, in comparison to the amount within a sample from a normal subject, has surprisingly been found to be indicative of a patient with a Fas-associated disease; this is true even though the mRNA levels themselves may not be diagnostic of disease. Particular diseases that may be diagnosed according to the serum levels of soluble Fas protein include certain human autoimmune diseases, particularly SLE and angioimmunoblastic lymphadenopathy (AILD).

The amount of a soluble Fas antigen present within a biological sample, such as blood, serum or PBMC sample,

may be determined by means of a molecular biological assay to determine the level of a nucleic acid that encodes such a mutant Fas antigen polypeptide, or by means of an immunoassay to determine the level of the polypeptide itself. Molecular biological assays still have many practical uses, even though they may not have diagnostic predictability in and of themselves. For example, in genetic engineering methods to express the useful soluble Fas antigen, molecular biological assays will be useful to confirm that the correct constructs are being manufactured and expressed.

In a molecular biological method for detecting a cell that produces a soluble Fas antigen, one would obtain nucleic acids from one or more cells and analyze the nucleic acids to identify a nucleic acid segment that encodes a Fas antigen that lacks a significant portion of the Fas antigen transmembrane region. Such nucleic acids may be identified by length, where an appropriate assay would be a PCR™-based assay resulting in the identification of a mutant transcript that is shorter in length than normal. Alternatively, the nucleic acid segment may be identified by sequence, which method generally includes identifying a transcript with a non-wild type sequence, *e.g.*, by Northern blotting using a discriminating probe.

To detect a cell that produces a soluble Fas antigen using a molecular biological method based upon the size of *fas* transcripts does not require a *fas* probe with a novel sequence. In such methods, any two *fas* primers from towards the termini of the wild type *fas* sequence may be employed to amplify, *e.g.*, using PCR™, the particular *fas* mRNA species from a given cell. The amplified products are then generally separated on the basis of size. The presence of a smaller sized mRNA transcript, or a significantly increased amount of such a

smaller sized transcript, in comparison to the amount within a normal or "control" cell or sample, is indicative of a cell that produces a soluble Fas antigen, or is indicative of a patient with SLE or AILD (as in FIG. 1).

In contrast, the detection of a cell that produces a soluble Fas antigen using a method based upon the sequence of a *fas* transcript requires a *fas* probe with a novel breakpoint spanning sequence, as disclosed herein. This imparts an evident utility to the nucleic acid segments of the present invention, particularly the shorter ones.

Further embodiments of the invention thus concern methods for detecting a cell that produces a mutant, soluble Fas antigen, based upon hybridization of unique *fas* sequences. To conduct such a method one would, generally, obtain sample nucleic acids from a sample containing cells, including a blood sample from a patient, and contact the sample nucleic acids with a nucleic acid segment capable of differentiating between soluble *fas* transcripts and wild type *fas* transcripts, under conditions effective to allow hybridization of substantially complementary nucleic acids. One would then detect the presence of any hybridized substantially complementary nucleic acid complexes that formed.

Nucleic acid segments or probes capable of differentiating between soluble *fas* transcripts and wild type *fas* transcripts will be those that include the novel sequences of the invention found to span the breakpoint region of the soluble *fas* transcript. Such nucleic acids will thus preferentially hybridize to soluble *fas* transcripts (*i.e.* those encoding a Fas polypeptide that lacks a transmembrane region) rather than wild type *fas* transcripts. It is expected that under certain readily-

identifiable conditions, depending on the extent of the additional 3' and 5' *fas* sequences surrounding the breakpoint region, these nucleic acid segments will exclusively bind to the alternatively-spliced mRNA transcripts discovered by the inventors. Thus, in general, shorter probes with sequences beginning in the area between about position 524 and about position 515 of SEQ ID NO:3 and ending in the area between about position 537 and about position 544 of SEQ ID NO:3, or equivalents thereof, are contemplated for use in these embodiments. However, should such sequences prove to bind to the wild-type *fas* mRNA, they may still be employed in discriminating hybridization, *e.g.*, by determining quantitative differences in hybridization or even by using gels that allow appropriate differentiation of the mRNA by size, with the soluble Fas-encoding sequences being smaller than the wild type sequences.

When using an appropriate *fas* probe with a unique breakpoint sequence, as described herein, the presence of a substantially complementary nucleic acid sequence in a sample, or a significantly increased level of such a sequence in comparison to the levels in a normal or "control" sample, will thus be indicative of a sample that contains a cell that harbors a soluble Fas antigen lacking a portion of the Fas transmembrane region. Here, substantially complementary soluble Fas nucleic acid sequences are those that have contiguous sequences with relatively little sequence divergence and that are capable of hybridizing under relatively stringent conditions, as discussed above.

Where a substantially complementary soluble Fas nucleic acid sequence, or significantly increased levels thereof, are detected in a clinical sample from a patient suspected of having an autoimmune disease, such as SLE or AILD, this will still likely be indicative of a patient

that has the disease. It should, however, be cautioned that mRNA detection assays are not proposed to be the assay of choice in diagnostic embodiments, rather it is currently preferred to determine soluble Fas levels as a diagnostic indicator. As used herein, the term "increased levels" is used to describe a significant increase in the amount of the soluble Fas nucleic acids detected in a given sample in comparison to that observed in a control sample, *e.g.,* an equivalent sample from a normal healthy subject.

A variety of hybridization techniques and systems are known that can be used in connection with the soluble Fas detection and/or disease diagnosis aspects of the invention, including diagnostic assays such as those described in Falkow *et al.*, U.S. Patent 4,358,535. Short nucleic acid segment probes from around the Fas breakpoint may also be employed as primers in connection with diagnostic PCR™ technology, as well as for use in any of a number of other PCR™ applications, including PCR™-based cloning and engineering protocols.

In general, the "detection" of a unique soluble Fas sequence is accomplished by attaching or incorporating a detectable label into the nucleic acid segment used as a probe and "contacting" a sample with the labeled probe. In such processes, an effective amount of a nucleic acid segment that comprises a detectable label (a probe), is brought into direct juxtaposition with a composition containing target nucleic acids. Hybridized nucleic acid complexes may then be identified by detecting the presence of the label, for example, by detecting a radio, enzymatic, fluorescent, or even chemiluminescent label.

Many suitable variations of hybridization technology are available for use in the detection of nucleic acids, as will be known to those of skill in the art. These

include, for example, *in situ* hybridization, Southern blotting and Northern blotting. *In situ* hybridization describes the techniques wherein the target or sample nucleic acids contacted with the probe sequences are located within one or more cells, such as cells within a clinical sample, or even cells grown in tissue culture. As is well known in the art, the cells are prepared for hybridization by fixation, *e.g.*, chemical fixation, and placed in conditions that allow for the hybridization of a detectable probe with nucleic acids located within the fixed cell.

Alternatively, the sample nucleic acids may be separated from a cell within a clinical sample prior to contact with a probe. Any of the wide variety of methods for isolating target nucleic acids may be employed, such as cesium chloride gradient centrifugation, chromatography (*e.g.*, ion, affinity, magnetic), phenol extraction and the like. Most often, the isolated nucleic acids will be separated, *e.g.*, by size, using electrophoretic separation, followed by immobilization onto a solid matrix, prior to contact with the labelled probe. These prior separation techniques are frequently employed in the art and are generally encompassed by the terms "Southern blotting" and "Northern blotting". The use of Northern blotting, in which the sample nucleic acids are RNA, is particularly preferred with the present invention. The execution of various techniques using labeled probes to detect alternatively spliced *fas* mRNA sequences in clinical samples will well known to those of skill in the art in light of the present disclosure.

Kits for use in molecular biological tests to identify transmembrane-lacking *fas* variants, and even to confirm the identity of individuals having, or being at risk for developing, systemic autoimmune diseases such as SLE and AILD, also fall within the scope of the present

invention. Such kits will generally comprise, in suitable container means, one or more *fas* nucleic acid probes or primers, one or more unrelated nucleic acid probes or primers for use as controls, and optionally, one or more restriction enzymes or PCR™ components. The components of the kits will preferably be packaged within distinct containers. *fas* nucleic acid probes capable of differentiating between soluble *fas* transcripts that lack a transmembrane domain encoding portion and wild type *fas* transcripts may be provided as the central component of a Northern blotting kit.

In still further embodiments, the present invention concerns the use of immunodetection methods for detecting a mutant or soluble Fas protein or polypeptide in a sample, including a serum samples from a patient that has, or is suspected to have, SLE, AILD or even another autoimmune disease. Human subjects with SLE have been discovered to have elevated serum levels of soluble Fas (Example IX, FIG. 6). It is contemplated that other disorders, such as ankylosing spondylitis, vasculitis and Sjogren's Syndrome, may also be diagnosed by detecting increased levels of soluble Fas.

The invention thus also provides immunodetection methods and associated kits. It is contemplated that the soluble Fas proteins and peptides of the invention may be employed to detect antibodies having reactivity therewith, or more preferably, that anti-Fas antibodies may be employed to detect soluble Fas. Kits are provided for use in the immunodetection of soluble Fas, and include kits for clinical diagnosis and kits for use in antigen or antibody purification or titering, as appropriate.

In general, immunodetection methods will include first obtaining a sample suspected of containing such a

soluble Fas protein or peptide, or anti-Fas antibody, such as a biological sample from a patient, and contacting the sample with a first soluble Fas protein or peptide, or a first antibody that binds to soluble Fas, as the case may be, under conditions effective to allow the formation of an immunocomplex (primary immune complex). One then detects the presence of any primary immunocomplexes so formed.

Contacting the chosen sample with the soluble Fas protein or peptide, or antibody thereto, under conditions effective to allow the formation of (primary) immune complexes is generally a matter of simply adding the protein, peptide or antibody composition to the sample. One then incubates the mixture for a period of time sufficient to allow the added antigens or antibodies to form immune complexes with, *i.e.* to bind to, any antibodies or antigens present within the sample. After this time, the sample composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antigen or antibody species, allowing only those specifically bound species within the immune complexes to be detected.

The detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches known to the skilled artisan and described in various publications, such as, *e.g.*, Nakamura *et al.* (1987), incorporated herein by reference. Detection of primary immune complexes is generally based upon the detection of a label or marker, such as a radioactive, fluorigenic, biological or enzymatic label, with enzyme tags such as alkaline phosphatase, horseradish peroxidase and glucose oxidase being suitable. The antigen or antibody employed may itself be linked to a detectable label, wherein one would

then simply detect this label, thereby allowing the amount of bound antigen or antibody present in the composition to be determined.

In one alternative, the primary immune complexes may be detected by means of a second binding ligand that is linked to a detectable label and that has binding affinity for the first protein, peptide or antibody. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labelled secondary antibodies or ligands, and the remaining bound label is then detected.

In yet another alternative, the secondary immune complexes may be detected by means of a tertiary binding ligand that is linked to a detectable label and that has binding affinity for the second binding ligand or antibody. The tertiary binding ligand will again often be an antibody, which may thus be termed a "tertiary" antibody. The secondary immune complexes are contacted with the labeled, tertiary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of tertiary immune complexes. The tertiary immune complexes are then generally washed to remove any non-specifically bound labelled antibodies or ligands, and the remaining bound label is then detected.

This latter alternative is exemplified by the currently preferred sandwich ELISA. Here, the anti-Fas antibodies are first immobilized and then contacted with

a soluble Fas sample, the secondary antibody is an unlabeled anti-Fas monoclonal antibody, and the tertiary antibody is a commercially available labeled antibody that is specific for a non-variant portion of the second monoclonal antibody.

For diagnostic purposes, it is proposed that virtually any sample suspected of containing a soluble Fas protein, or even an anti-Fas antibody sought to be detected, as the case may be, may be employed. Exemplary samples include clinical samples obtained from a patient, such as blood or serum samples, PBMCs, or even perhaps spleen samples. Furthermore, it is contemplated that such embodiments may have application to non-clinical samples, such as in the titering of antigen or antibody samples, in the selection of hybridomas, and the like.

In related embodiments, the present invention contemplates the preparation of kits that may be employed to detect the presence of a soluble Fas proteins or peptides and/or anti-Fas antibodies in a sample. Generally speaking, kits in accordance with the present invention will include a suitable a soluble Fas protein or peptide, or a first antibody that binds to a soluble Fas protein or peptide, together with an immunodetection reagent, and a means for containing the protein, peptide or antibody and reagent.

The immunodetection reagent will typically comprise a label associated with the Fas protein or peptide or anti-Fas antibody, or associated with a secondary binding ligand. Exemplary ligands might include a secondary antibody directed against the first Fas protein or anti-Fas antibody, or a biotin or avidin (or streptavidin) ligand having an associated label. Detectable labels linked to antibodies that have binding affinity for a human antibody are also contemplated, *e.g.*, for protocols

where the first reagent is a Fas protein that is used to bind to a reactive antibody from a human sample. Of course, as noted above, a number of exemplary labels are known in the art and all such labels may be employed in connection with the present invention. The kits may contain Fas antigen or antibody-label conjugates either in fully conjugated form, in the form of intermediates, or as separate moieties to be conjugated by the user of the kit.

The container means will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the antigen or antibody may be placed, and preferably suitably allocated. Where a second, or even a second and a third, binding ligand is provided, the kit will also generally contain a second, or even a second and a third, vial or other container into which these ligand(s) or antibody(ies) may be placed. The kits of the present invention will also typically include a means for containing the vials in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials are retained.

More specifically, the preferred kits in accordance with the present invention will include a first antibody that binds to a soluble Fas antigen polypeptide that lacks a transmembrane domain; a suitably aliquoted composition of said mutant Fas antigen polypeptide; and an immunodetection means. Even more preferably, the first antibody will be bound to a solid support, such as ELISA plate.

In still further embodiments, the discoveries of the present invention are contemplated for use in designing new treatment strategies for autoimmune diseases. For example, drugs may be identified or designed to normalize

transcription of genes important in tolerance induction and apoptosis, particularly the *fas* gene, but also other apoptosis genes known to those of skill in the art, and to restore normal gene function despite the presence of a mutation. Agents may also be identified or designed to normalize protein function, or protein levels, irrespective of ongoing abnormal gene function.

To treat an autoimmune disease or other disorder associated with aberrant apoptosis, it is contemplated that one would administer to a patient with such a disease or disorder an immunologically effective amount of a pharmaceutically acceptable composition capable of promoting or restoring normal apoptosis. To increase apoptosis from an inappropriately low level, as required to treat autoimmune diseases, malignancy or to combat aging, one may administer an agent, such as anti-Fas antibody, to eliminate excess soluble Fas. To decrease apoptosis from an inappropriately high level, as required to treat strokes or heart attacks, one may administer an agent, including soluble Fas itself, that results in raising the serum levels of soluble Fas. All such methods are thus encompassed by the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. RT-PCR™ analysis of the human *fas* mRNA species. First-strand cDNA was prepared from the human PBMC as indicated in Example I and subjected to PCR™ using primers corresponding to sequences in the 5′- and 3′-

untranslated region (UTR) of *fas* mRNA. Specifically, the primers are 5'-CACTTCGGAGGATTGCTCAACA-3', (nucleotides 170-191; position 1-22 of SEQ ID NO:1) and 5'-TATGTTGGCTCTTCAGCGCTA-3', SEQ ID NO:11 (complementary to nucleotides 1316-1336). The PCR™ products were separated in a 1.2% agarose gel and stained with ethidium bromide. Standard DNA size markers are shown to the right of the gel.

FIG. 2. Agarose gel electrophoreses of *EcoR I* digests of representative recombinants. The recombinants were derived by cloning DNA fragments amplified from the normal subject 1, shown for comparison in the first lane of the gel. The DNA fragments were eluted from a gel fragment corresponding to the region indicated by the bracket to the left of the gel. The five cloned inserts and the number of bp in each insert are *fas* cDNA, -1167 bp insert; FasΔ1, -1104 bp insert; FasΔ2, -975 bp insert; FasΔ3 -920 bp insert; FasΔ4 -857 bp insert. These correspond to the DNA sequences SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9, respectively. Standard DNA size markers are shown to the right of the gel.

FIG. 3. A schematic representation of alternatively spliced *fas* mRNA variants. The translated- and UTR regions are indicated by boxes and thick solid lines, respectively. Regions lacking in the *fas* mRNA variants are indicated by broken lines and nucleotide positions. LP, CR, TM, ST, and NR represent leader peptide, cysteine-rich subdomains, transmembrane domain, signal transduction domain, and negative regulation domain, respectively (Itoh *et al.*, 1991; Oehm *et al.*, 1992; Itoh and Nagata, 1993). Nucleotide regions encoding each domain are shown at the top (Itoh *et al.*, 1991; Itoh and Nagata, 1993). AL indicates the altered amino acid sequences in the *fas* mRNA variants.

FIG. 4. Inhibition assay for apoptosis induced by anti-Fas Ab. Normal human PBMC were stimulated with PHA-P and then incubated in RPMI 1640 with 200 μg/ml murine anti-human Fas MAb in presence of various concentrations of supernatant harvested from COS cells transfected with either full-length *fas* cDNA or FasΔ1. Cells undergoing apoptosis were determined by the TdT method (Gavrieli *et al.*, 1992). A total of 200 cells including apoptotic cells and live cells were counted in 10 randomly selected microscopic fields. The percentage of viable cells not undergoing apoptosis is indicated. *fas* cDNA, open squares; FasΔ1, filled diamonds.

FIG. 5. Schematic diagram representing the *fas* mRNA region of the present invention. The *fas* probe and the probe predicted to be protected by the *fas* mRNA and the FasΔTM variant are shown. The transcription start site for SP6 transcript and the *EcoR* I restriction site used for linearization of the recombinants are indicated. Sequence corresponding to the alternatively spliced exon is indicated by a solid box.

FIG. 6. Soluble Fas in SLE patients. Sera from control subjects, SLE and rheumatoid arthritis (RA) patients were analyzed by ELISA for soluble Fas. Approximately 60% of SLE patients have elevated soluble Fas levels. Plates were coated with anti-human Fas MAb (1 μg/ml) blocked with 1% BSA. 100 μl of the sera (1:40 diluted in 1% BSA) was added in duplicate to microtiter plate wells. Polyclonal rat anti-human Fas sera, diluted 1:100 in 1% BSA was used to detect the bound human Fas, and this was detected using an anti-rat IgG alkaline phosphatase antibody. A positive control was a human Fas fusion protein. A negative control was a control supernatant.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors herein demonstrate the presence of four human Fas mRNA variants that are derived by alternative splicing of the Fas gene transcripts. The coding regions that are deleted corresponded to nucleotides 700-762 in the variant FasΔ1; 529-637 and 763-845 for FasΔ2; 391-637 for FasΔ3; and 391-637 and 700-762 for FasΔ4. All four variants are capable of encoding truncated Fas proteins that lack the transmembrane domain due either to the deletion of the nucleotides encoding this region (FasΔ1 and FasΔ4), or deletion of nucleotides resulting in insertion of an in-frame termination codon in this region (FasΔ2 and FasΔ3). The putative translation products encoded by the variants FasΔ2, FasΔ3 and FasΔ4 lack the middle portion of the extracellular domain and have an altered transmembrane region and much shorter carboxyl-terminal (FIG. 3). It is possible that these polypeptides can be secreted, but it is unlikely that they interact with the Fas ligand as they probably are unable to retain the native conformation of the extracellular domain.

It should be noted that norther blot analysis gave rise to two prominent bands at approximately 2.7- and 1.9-kb, respectively. The two mRNA species differing in size of an approximately 0.8 kb are likely due to differences in the length of the 3'-UTR as a results of alternative use of two polyadenylation signals (Itoh *et al.*, 1991) and cannot be due to the variant Fas mRNA identified in PBMC as none of the variants have a nucleotide deletion that is sufficient to account for the 0.8 kb difference. That alternative splicing of exons likely gives rise to FasΔ1 (63 bp deletion), that cannot be distinguished from the full-length Fas on northern blot, and FasΔ2, FasΔ3 and FasΔ4, that may be of

insufficient abundance to be detected by norther blot analysis.

The FasΔ1 variant (FIG. 3) expressed the putative translation product containing the leader peptide, most residues of the extracellular domain, the cytoplasmic tail and two potential N-linked glycosylation sites, but lacked the entire transmembrane domain except the final Val, and five residues immediately up-stream of the transmembrane domain. These alterations may prevent retention of the Fas molecule in the plasma membrane and consequently, could result in the production of a secreted Fas antigen. The possibility that the Fas antigen may also be expressed as a soluble product was confirmed by comparative flow cytometry and immunocytochemical analysis of COS cells transfected either with the FasΔ1 variant or the full-length cDNA. Supernatants obtained from the FasΔ1 transfected COS cells were able to block anti-Fas antibody-induced apoptosis in activated PBMC cells in a dose-dependent fashion. This is consistent with previous description of a secreted inhibitor of apoptosis (Wylliams *et al.*, 1990).

The inventors further cloned and sequenced a *fas* genomic DNA isolated from a human placenta genomic library. This also resulted in the identification of the FasΔ1 mRNA variant (also designated FasΔTM), that is capable of encoding a truncated Fas protein that lacks the transmembrane domain due to the deletion of an exon encoding this region. The existence of the FasΔ1 (FasΔTM) transcript in normal human PBMC was confirmed by RNase protection analysis. It is thought that this form of Fas is produced by exon-skipping, on the basis of genomic DNA analysis.

The existence of its encoded soluble form of the Fas antigen was again demonstrated in the cytoplasm of the transfected COS-7 cells by protein expression studies. Supernatants obtained from COS-7 cells that transiently expressed FasΔ1 were also able to block anti-Fas antibody-induced apoptosis in activated PBMC cells, providing further evidence that this soluble protein is secreted.

The finding that the Fas apoptosis molecule can exist in a secreted form is proposed to be of physiological significance since a secreted molecule that is capable of ligand binding could compete with the surface expressed Fas molecule for interaction with the Fas ligand. This would inhibit Fas antigen-mediated apoptosis. In this regard, increased production of soluble forms of Fas antigen may contribute to the etiology and development of human autoimmune diseases, including SLE and angioimmunoblastic lymphadenopathy (AILD).

The RT-PCR™ analysis (FIG. 1) revealed differences in the relative amount of the PCR™ fragments between the normal controls and patients with SLE and with AILD. The predominant *fas* RT-PCR™ products in the normal controls correspond to full-length *fas* mRNAs, whereas in two SLE and one AILD patient, the predominant RT-PCR™ product is the *fas* message encoding the transmembrane (63 bp) deletion mutant.

Also, in light of the fact that Fas-mediated apoptosis has been implicated in lymphocyte apoptosis in HIV infection, the Fas mutants may have a role in other immunodeficiencies. It is proposed that a blockade of Fas-mediated apoptosis *in vivo*, using the recombinant soluble Fas of the present invention, may prove to be of clinical utility in such disease conditions.

Additionally, the inventors (Su *et al.*, 1994) and others (Tsui *et al.*, 1993, Schultz *et al.*, 1993) have recently isolated a phosphatase gene that is involved in regulation of either apoptosis or Fas expression. Novel therapies might be designed that alter soluble Fas and regulate apoptosis.

1. Biological Functional Equivalents

Modification and changes may be made in the structure of the soluble Fas proteins and peptides and still obtain a molecule having like or otherwise desirable characteristics. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules, ligands or receptors. Since it is the interactive capacity and nature of a protein or peptide that defines that protein or peptide's biological functional activity, certain amino acid sequence substitutions can be made in a protein or peptide sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a protein or peptide with like (agonistic) properties. Equally, the same considerations may be employed to create a protein or polypeptide with countervailing (*e.g.*, antagonistic) properties. It is thus contemplated by the inventors that various changes may be made in the sequence of the soluble Fas proteins or peptides (or underlying DNA) without appreciable loss of their biological utility or activity.

It is also well understood by the skilled artisan that, inherent in th definition of a biologically functional equivalent protein or peptide, is the concept that there is a limit to the number of changes that may

be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted. In particular, where smaller length peptides are concerned, it is contemplated that only a few amino acids may be changed within a given peptide. Of course, a plurality of distinct proteins/peptides with different substitutions may easily be made and used in accordance with the invention.

It will also be well understood that, in the context of the present invention, the contiguous Fas peptide sequence spanning the region from which the transmembrane portion has been deleted are particularly important to the novelty of the soluble Fas protein or peptide. As such, a substantial number of additional residues cannot generally be added to this region and still result in the type of truncated Fas molecules particularly contemplated by the inventors.

In making such changes of an equivalent nature, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and

Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, keep the next portion for immunological stuff particularly where the biological functional equivalent protein or peptide thereby created is intended for use in immunological embodiments, as in the present case. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *i.e.* with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those

which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

Amino acid substitutions are thus generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

While discussion has focused on functionally equivalent polypeptides arising from amino acid changes, it will be appreciated that these changes may be effected by alteration of the encoding DNA; taking into consideration also that the genetic code is degenerate and that two or more codons may code for the same amino acid. A table of amino acids and their codons is presented hereinbelow for use in such embodiments, as well as for other uses, such as in the design of probes and primers and the like.

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In addition to the Fas peptide compounds described herein, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the peptide structure. Such compounds, which may be termed peptidomimetics, may be used in the same manner as the peptides of the invention and hence are also functional equivalents. The generation of a structural functional equivalent may be achieved by the techniques of modelling and chemical

design known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

U.S. Patent 4,554,101 (Hopp, incorporated herein by reference) also teaches the identification and preparation of epitopes from primary amino acid sequences on the basis of hydrophilicity. Through the methods disclosed in Hopp one of skill in the art would be able to identify epitopes from within an amino acid sequence such as the Fas sequences disclosed herein. These regions are also referred to as "epitopic core regions".

Numerous scientific publications have been devoted to the prediction of secondary structure, and to the identification of epitopes, from analyses of amino acid sequences (Chou and Fasman, 1974a,b; 1978a,b, 1979). Any of these may be used, if desired, to supplement the teachings of Hopp in U.S. Patent 4,554,101. Moreover, computer programs are currently available to assist with predicting antigenic portions and epitopic core regions of proteins. Examples include those programs based upon the Jameson-Wolf analysis (Jameson and Wolf, 1998; Wolf *et al.*, 1988), the program PepPlot® (Brutlag *et al.*, 1990; Weinberger *et al.*, 1985), and other new programs for protein tertiary structure prediction (Fetrow and Bryant, 1993).

2. Monoclonal Antibody Generation

Means for preparing and characterizing antibodies are well known in the art (See, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for

preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic composition in accordance with the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide, such as Fas-derived peptide, to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimyde and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for

immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, a purified or partially purified soluble Fas protein, polypeptide or breakpoint-spanning peptide. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because

peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984). cites). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.* (1977). The use of electrically induced fusion methods is also appropriate (Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1 X 10^{-6} to 1 X 10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway,

but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

If desired, antibodies in accordance with the present invention may be specifically selected on the basis of only binding to the region of a soluble Fas protein or peptide that bridges the region from which the Fas transmembrane sections have been deleted. This is a simply a matter of conducting comparative binding assays between the candidate antibodies and native Fas or the Fas proteins and peptides disclosed herein. As the Fas compounds identified herein have novel contiguous peptide sequences it is likely that unique MAbs will be generated against such soluble Fas peptides and polypeptides.

In any event, the selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal

develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

3. Unique Fas Epitopes

The present invention encompasses anti-Fas antibodies and antibody-based compositions, such as antibody conjugates, that bind to unique epitopic sites on Fas peptides and proteins that have been created as a result of the novel alternative splicing described herein. Such anti-Fas antibodies may be of the polyclonal or monoclonal type, with monoclonals being generally preferred.

The soluble Fas antigen polypeptides described herein each include a novel contiguous sequence which links elements of the intracellular and extracellular domains. This is exemplified by the contiguous amino acid sequences Lys Cys Lys Glu Glu Val Lys Arg Lys Glu (position 164 to 173 of SEQ ID NO:4) and Leu Thr Ser Asn Thr Lys Cys Lys Glu Glu Val Lys Arg Lys Glu Val Gln Lys Thr Cys (position 159 to 178 of SEQ ID NO:4) and, indeed, all peptides of between about 7 and about 20 amino acids in length that include the sequence Lys Glu Glu Val Lys Arg in combination with contiguous N- or C-terminal sequences from the immediately adjoining Fas regions. Antibodies that bind to these and similar peptides thus

fall within the scope of this invention, as do "cross-hybridizing antibodies".

The identification of an antibody that binds to an epitope substantially the same as the epitopes spanning the junction of the Fas intracellular and extracellular domains is a fairly straightforward matter. This can be readily determined using any one of variety of immunological screening assays in which antibody competition can be assessed. For example, where the test antibodies to be examined are obtained from different source animals, or are even of a different isotype, a simple competition assays may be employed in which the control and test antibodies are premixed and then applied to an antigen composition. By "antigen composition" is meant any composition that contains a soluble Fas antigen as described herein. Thus, protocols based upon ELISAs and Western blotting are suitable for use in such simple competition studies.

In such embodiments, one would pre-mix the control antibodies with varying amounts of the test antibodies (*e.g.*, 1:1, 1:10 and 1:100) for a period of time prior to applying to an antigen composition, such as an antigen-coated well of an ELISA plate or an antigen adsorbed to a membrane (as in dot blots and Western blots). By using species or isotype secondary antibodies one will be able to detect only the bound control antibodies, the binding of which will be reduced by the presence of a test antibody which recognizes the same epitope/antigen.

In conducting an antibody competition study between control antibody and any test antibody, one may first label the control with a detectable label, such as, *e.g.*, biotin or an enzymatic, radioactive or fluorogenic label, to enable subsequent identification. In these cases, one would incubate the labelled control antibodies with the

test antibodies to be examined at various ratios (*e.g.*, 1:1, 1:10 and 1:100) and, after a suitable period of time, one would then assay the reactivity of the labelled control antibodies and compare this with a control value in which no potentially competing test antibody was included in the incubation.

The assay may again be any one of a range of immunological assays based upon antibody hybridization, and the control antibodies would be detected by means of detecting their label, *e.g.*, using streptavidin in the case of biotinylated antibodies or by using a chromogenic substrate in connection with an enzymatic label or by simply detecting a radioactive or fluorescent label. An antibody that binds to the same epitope as the control antibodies will be able to effectively compete for binding and thus will significantly reduce control antibody binding, as evidenced by a reduction in bound label.

The reactivity of the labelled control antibodies in the absence of any test antibody would be the control high value. The control low value would be obtained by incubating the labelled antibodies with unlabelled antibodies of the same type, when competition would occur and reduce binding of the labelled antibodies. A significant reduction in labelled antibody reactivity in the presence of a test antibody is indicative of a test antibody that recognizes the same epitope, *i.e.* one that "cross-reacts" with the labelled antibody. A significant reduction is a reproducible, *i.e.* consistently observed, reduction in binding.

4. ELISAs

An ELISA assay may be used to detect soluble Fas, *e.g.*, in the sera of patients with autoimmune diseases. Various ELISA methods may be employed, as described by Harlow and David (1988; incorporated herein by reference). In one such ELISA, peptides incorporating the unique Fas antigen sequences of invention may be first immobilized onto a selected surface, *e.g.*, a well of a surface exhibiting a protein affinity, such as a well in a polystyrene microtiter plate. In such an ELISA, generally, labelled anti-Fas antibodies would then be added to the wells, allowed to bind, and detected by means of their label. The amount of soluble Fas in a sample (unknown) would be determined by mixing the sample with the labelled anti-Fas antibodies before or during incubation with the Fas in the wells. The presence of soluble Fas in the sample acts to reduce the amount of anti-Fas antibody available for binding to the well and thus reduces the ultimate signal.

In another form of ELISA, an antibody capable of binding a soluble Fas protein or peptide of the invention may be immobilized onto the solid surface, or well, and used directly in conjunction with labelled Fas compositions. In these ELISAs, generally, labelled Fas is added to the wells, allowed to bind, and detected by means of the label. The amount of soluble Fas in any given sample is here determined by mixing the sample with the labelled Fas before or during incubation with the anti-Fas antibody in the wells. The presence of soluble Fas in the sample again acts to reduce the amount of labelled Fas available for binding to the well and thus reduces the ultimate signal.

In coating a plate with either antigen or antibody, one will generally wash the wells of the plate to remove

incompletely adsorbed material and then bind or "coat" a nonspecific protein onto the wells of the plate. Nonspecific proteins are those that are known to be antigenically neutral with regard to the test antisera, and include bovine serum albumin (BSA), casein and solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

Where an antibody capable of binding a soluble Fas protein is immobilized onto an ELISA plate, it is more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the control Fas and/or clinical or biological sample to be tested in a manner conducive to immune complex (antigen/antibody) formation. Detection of the Fas then requires a labelled secondary antibody, or a secondary antibody and a labelled tertiary, antibody. The labelled secondary antibody is, of course, an anti-Fas antibody that is conjugated to a detectable label. When using a tertiary approach, the secondary antibody is an unlabelled anti-Fas antibody and the tertiary antibody is a labelled antibody that is specific for the species, or isotype, of the secondary antibody employed.

A "manner conducive to immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background.

Incubation steps are typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25° to 27°C, or may be overnight at about 4°C or so. Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-immunocomplexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween®, or borate buffer.

Following the formation of specific immunocomplexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immunocomplexes may be determined. As mentioned above, this may be achieved by subjecting the first immunocomplex to a second antibody having specificity for the first, or even a third antibody having specificity for the second. Where a second antibody alone is used, given that the control and test Fas samples will typically be of human origin, the second antibody will preferably be an antibody having specificity in general for human Fas. Where a third antibody is also used, the second antibody will still preferably be an antibody having specificity for human Fas, and the third antibody will then be an antibody having specificity in general for the second antibody. A second murine antibody and a third anti-mouse Ig antibody is a particular example.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immunocomplex with a urease, glucose oxidase or peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immunocomplex formation (*e.g.*, incubation for 2 hours at

room temperature in a PBS-containing solution such as PBS-Tween®).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid [ABTS] and H_2O_2, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra spectrophotometer. A specific example of a soluble Fas ELISA is described in Example VIII.

5. Nucleic Acid Hybridization

The use of a Fas breakpoint-spanning hybridization probe of about 10-14 nucleotides in length allows the formation of a duplex molecule that is both stable and selective and is thus able to identify like nucleic acids, *e.g.*, those located within cells that have alternative Fas splicing. Molecules having contiguous complementary sequences over stretches greater than 10 bases in length are generally preferred in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 20 contiguous nucleotides, or even longer where desired.

Hybridization probes may of course be selected from any portion of any of the sequences disclosed herein. All that is required is to revi w the sequence set forth and to select any continuous portion of the sequence, from about 10/14, or preferably about 14, nucleotides in length up to and including the full-length sequence, that

one wishes to utilize as a probe or primer. The choice of probe and primer sequences may be governed by various factors, such as, by way of example only, one may wish to employ primers from towards the termini of the total sequence, or from the ends of the functional domain-encoding sequences, in order to amplify further DNA; one may employ probes corresponding to the entire DNA to clone Fas genes from other species or to clone further Fas-like or homologous genes from any species including human; and one may employ mutant probes or primers in site specific mutagenesis.

Naturally, the preferred hybridization probes will be selected from the contiguous sequences disclosed that link, for the first time, the intracellular and extracellular Fas domains without having any intervening coding sequence for the Fas transmembrane domain. This is exemplified by contiguous sequences that span the breakpoint, *e.g.*, from position 526 to 535 of SEQ ID NO:3, from position 524 to 537 of SEQ ID NO:3, or from position 520 to 540 of SEQ ID NO:3, from position 515 to 544 of SEQ ID NO:3, and, indeed, all sequences of between about 10-14, about 20, up to and including and about 60 or so nucleotides in length that include about a 10-14 bp sequence that spans the breakpoint, optionally in combination with contiguous 5′ or 3′ sequences from the immediately adjoining Fas regions.

The process of selecting and preparing a nucleic acid segment that includes a contiguous sequence from this sequence region may alternatively be described as preparing a nucleic acid fragment. Of course, fragments may also be obtained by other techniques such as, *e.g.*, by mechanical shearing or by restriction enzyme digestion. Small nucleic acid segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is

commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as the PCR™ technology of U.S. Patent 4,683,202 (incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of Fas cDNAs or mRNAs. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and\or high temperature conditions, such as provided by 0.02M-0.15M NaCl at temperatures of 50°C to 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating or identifying alternatively spliced mRNA.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate Fas-encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desir to employ conditions such as 0.15M-0.9M salt, at temperatures ranging from 20°C to 55°C. Cross-hybridizing species can

thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the

particular circumstances based on the particular criteria required (depending, for example, on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

6. Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art as exemplified by publications (Adelman *et al.*, 1983). As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (Messing *et al.*, 1981). These phage are readily

commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart the two strands of a double stranded vector which includes within its sequence a DNA sequence which encodes the Fas antigen. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example by the method of Crea *et al.* (1978). This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected Fas antigen using site-directed mutagenesis is provided as a means of producing potentially useful Fas species and is not meant to be limiting as there are other ways in which sequence variants of Fas may be obtained. For example, recombinant vectors encoding the desired Fas gene may be treated with mutagenic agents to obtain sequence variants (see, *e.g.*, a method described by Eichenlaub, 1979) for the mutagenesis of plasmid DNA using hydroxylamine.

7. **Recombinant Host Cells**

Suitable prokaryotic host cells for use with the present invention include *E. coli*. In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, which contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, the phage lambda GEM^{TM}-11 may be utilized in making a recombinant phage vector which can be used to transform host certain *E. coli* cells.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used. This plasmid already contains the *trpl* gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1. The presence of the *trpl* lesion as a characteristic of the

yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, an origin of replication, and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years. Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7, 293 and MDCK cell lines. Expression vectors

for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

The origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (*e.g.*, Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

8. Pharmac utical Compositions

As soluble Fas, anti-Fas antibodies and other agents are contemplated for use in treating diseases connected with aberrant apoptosis, such agents may be formulated into pharmaceutically acceptable compositions for administration to a patient.

Aqueous compositions of the present invention will comprise an effective amount of the soluble Fas or anti-Fas agent, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The active compounds may also be formulated for parenteral administration, *e.g.*, formulated for injection *via* the intravenous, intramuscular or sub-cutaneous routes. The preparation of an aqueous composition that contains soluble Fas or anti-Fas agent as an active ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for

using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Soluble Fas or an anti-Fas agent such as an antibody can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as

isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus ny additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 mL of isotonic NaCl solution and either added to 1000mL of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

In addition to the compounds formulated for parenteral administration, other pharmaceutically acceptable forms include, *e.g.*, tablets or other solids for oral administration; time release capsules; and any other form currently used, including cremes, lotions, mouthwashes, inhalents and the like.

In certain embodiments, active Fas or anti-Fas compounds may even be administered orally. This is

contemplated for agents that are generally resistant, or have been rendered resistant, to proteolysis by digestive enzymes. Such compounds are contemplated to include chemically designed or modified agents; dextrorotatory peptides; and peptide and liposomal formulations in time release capsules to avoid peptidase and lipase degradation.

For oral administration, the active compounds may be administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or compressed into tablets, or incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to

otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I

Identification of Human *fas* Gene Deletion Mutants in PBMC

A. Methods

1. Isolation of PBMC. Peripheral blood was drawn from normal control subjects or from patients referred to the National Institutes of Health and meeting the criteria for SLE or AILD (Cohen *et al.*, 1971). PBMC were separated from freshly drawn heparinized venous blood by

Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation.

2. **Isolation of RNA**. Total cellular RNA was isolated from the cells by the guanidine isothiocyanate/acid phenol method (Chomcynski and Sacchi, 1987).

3. **Northern Blot Analysis.** Samples of total cellular RNA (20 μg) were fractionated in a formaldehyde denaturing gel, and transferred onto nitrocellulose filters (Microseparations, Inc., Westboro, MA) (Fourney *et al.*, 1988). The filters were pre-hybridized for 4 h and hybridized for 16 h at 42°C on a shaking incubator in buffer containing 50% formamide, 5x Denhardt's, 1 M NaCl, 10% Dextran sulfate, 50 mM Tris-HCl, pH 7.4, 0.1% SDS, and 100 μg/ml sonicated salmon sperm DNA. After hybridization the filters were washed in 2xSSC, 0.1% SDS at room temperature for 30 min, and then in 0.2xSSC, 0.1% SDS at 65°C for 1 h. Autoradiography was carried out at - 70°C between two intensifying screens for 10 days.

4. **cDNA Probe.** The probe used was a 1167 base pair (bp; SEQ ID NO:1) *fas* cDNA fragment derived from RT-PCR™ using 5' and 3' primers for the human *fas* cDNA and cloned in the *EcoR I* site of the pCR™ vector (Invitrogen, San Diego, CA). The insert was excised from the plasmid by digestion with *EcoR I* and electrophoresed on a 0.8% low-temperature melting agarose gel (NuSieve GTG agarose, MFC Corp., Rockland, ME). The probe was labeled with [α-^{32}P]dCTP by using the random hexanucleotide priming method (Feinberg and Vogelstein, 1984).

5. **Oligonucleotide Synthesis**. Primers were synthesized on an automated DNA/RNA synthesizer Model 394 (Applied Biosystems, Foster, CA).

6. Rev rs Transcription PCR™ (RT-PCR™). The first-strand cDNA syntheses were carried out using the first-strand cDNA synthesis kit (Pharmacia P-L Biochemicals Inc., Milwaukee, WI) by the recommended procedure. 10 μg of total cellular RNA and oligo dT were used for the cDNA synthesis in a final volume of 20 μl by reverse transcriptase. Base pairs are numbered throughout this disclosure as described in Itoh *et al* (1991).

Two oligonucleotides, 5'-CACTTCGGAGGATTGCTCAACA-3', (nucleotides 170-191; position 1-22 of SEQ ID NO:1) and 5'-TATGTTGGCTCTTCAGCGCTA-3', SEQ ID NO:11 (complementary to nucleotides 1316-1336), were used to amplify human *fas* mRNA from nucleotide 170 to nucleotide 1336 (SEQ ID NO:1). The amplification was performed in a 100 μl reaction volume using 0.2 μg template and 2.5 units of Taq DNA polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk, CT). The PCR™ was carried out for a total of 30 cycles using a TempCycle (Perkin-Elmer Cetus, Norwalk, CT). Each cycle consisted of denaturation at 94°C for 60 seconds, annealing at 56°C for 90 seconds and extension at 72°C for 120 seconds.

7. PCR™ Product Cloning. The PCR™ products were analyzed electrophoretically on 1.2% agarose gels (Seakem GTG agarose, FMC Corp.) and recovered from 0.8% low-temperature melting agarose gels (NuSieve GTG agarose, FMC Corp.). The products were directly subcloned into a pCR™ vector (Invitrogen, San Diego, CA) following the procedures recommended by the supplier.

E. coli cells were transformed with the vectors containing these DNA fragments and plated. Sixteen transformants were randomly selected from approximately 10^4 colonies to prepare plasmid DNA. The inserts of

these 16 plasmids were excised by *Eco RI* and electrophoresed in a 1.2% agarose gel.

8. Nucleotide Sequencing and Analysis. The templates for nucleotide sequencing were alkali-denatured plasmids that were purified using QIAGEN™ 100-tips (QIAGEN Inc., Studio, CA) following the procedure recommended by the supplier. All nucleotide sequences were determined by the dideoxy chain termination method using modified bacteriophage T7 DNA polymerase (Smith *et al.*, 1989) (United States Biochemical Corp., Cleveland, OH).

Oligonucleotides, Sp6 and T7 (Invitrogen, San Diego, CA), and an additional two, 5'AACTGCACCCGGACC-3', (nucleotides 546-560; position 377 to position 391 of SEQ ID NO:1) and 5'-TGTGTCATACGCTTC-3', SEQ ID NO:12 (complementary to nucleotides 1059-1076), derived from the *fas* cDNA sequence, were used to obtain the overlapped sequence. Nucleotide sequencing data were analyzed by the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group (Madison, WI) and the MacVector Sequence Analysis Software (International Biotechnologies, Inc., New Haven, CT).

B. Results

To investigate the possible presence of abnormal *fas* transcripts in SLE and AILD patients, the inventors isolated cellular RNA from the human PBMC obtained from two SLE and two AILD patients, and three normal controls and performed northern blot analysis for direct visualization of the *fas* mRNA species. Both the normal controls and the patients exhibited two distinct transcripts with similar sizes to those previously reported (Itoh *et al.*, 1991). There was no detectable difference in size of the *fas* transcripts in normal individuals compared to the SLE and AILD patients.

The *fas* mRNA transcripts were then analyzed after amplification using oligonucleotide primers derived from the 5'-untranslated region (UTR), starting at nucleotide 170, and 3'-UTR of the *fas* mRNA (Itoh *et al.*, 1991) (FIG. 1). The amplified product was expected to be 1167-bp in size, and to encompass the entire translation region, as well as portions of the 5'- and 3'-UTR of the *fas* mRNA. However, in addition to the expected full-length *fas* cDNA fragment, 4 distinct smaller DNA fragments were also observed. It is important to note that, in some patients, the relative intensity of the full-length *fas* mRNA was decreased compared with the second largest *fas* mRNA fragments.

To determine the structure and significance of the five prominent PCR™ products, which ranged in size from 1167-base pairs to 857-base pairs, DNA fragments were recovered from the corresponding gel segment of normal control #1 and subcloned into the pCR™ vector (FIG. 2). From approximately 10^4 colonies, five discrete DNA inserts were isolated, referred to herein as *fas* cDNA (1167 base pairs), FasΔ1 (1104 base pairs), FasΔ2 (975 base pairs,), FasΔ3 (920 base pairs), and FasΔ4 (857 base pairs).

Comparison of the nucleotide and the deduced amino acid sequences of the four *fas* mRNA variants to the sequences of the full-length human *fas* was performed. Both the nucleotide and the deduced amino acid sequences of the four mRNA variants were aligned to the sequences of the full-length Fas (Itoh *et al.*, 1991). Primer positions for amplification of the *fas* mRNA were from nucleotides 170-191 (position 1-22 of SEQ ID NO:1) and a primer complementary to nucleotides 1316-1336 (SEQ ID NO:11).

The nucleotide sequence of the full-length *fas* from position 170 to position 1336 is SEQ ID NO:1 (1167 base pairs), and the corresponding deduced amino acid sequence is SEQ ID NO:2 (335 amino acids, including the 319 of the soluble protein and the 16 residue underlined leader peptide). The nucleotide sequence of FasΔ1 is SEQ ID NO:3 (1104 base pairs), and the corresponding deduced amino acid sequence is SEQ ID NO:4 (314 amino acids, including the 298 of the soluble protein and the 16 residue underlined leader peptide). The nucleotide sequence of FasΔ2 is SEQ ID NO:5 (975 base pairs), and the corresponding deduced amino acid sequence is SEQ ID NO:6 (165 amino acids, made up of 149 and the 16 residue underlined leader peptide). The nucleotide sequence of FasΔ3 is SEQ ID NO:7 (920 base pairs), and the corresponding deduced amino acid sequence is SEQ ID NO:8 (119 amino acids, made up of 103 and the 16 residue leader peptide). The nucleotide sequence of FasΔ4 is SEQ ID NO:9 (857 base pairs), and the corresponding deduced amino acid sequence is SEQ ID NO:10 (102 amino acids, including 86 and the 16 residue leader peptide).

The nucleotide sequence derived from each insert, and the deduced amino acid sequence, was compared to that of the previously reported human *fas* (Itoh *et al.*, 1991). A schematic representation of the pattern of four alternatively spliced *fas* mRNA sequences is shown in FIG. 3. The nucleotide sequence comparison confirmed that the longest insert (shown in *fas* cDNA of FIG. 2) represented the expected 1167-base pair *fas* cDNA fragment corresponding to nucleotides 170 to 1336 (SEQ ID NO:1) and contained the intact open reading frame that encoded a protein with the amino acid sequence of SEQ ID NO:2. The remaining inserts displayed distinct internal nucleotide deletions.

Specifically, FasΔ1 (SEQ ID NO:3), has a 63-base pair deletion starting at nucleotide sequence 700-GA TCC AGA and ending at nucleotide sequence GTT TGG G-762. This deletion does not alter the authentic downstream *fas* reading frame, and therefore, FasΔ1 is capable of encoding a truncated Fas protein product. The putative translation product (SEQ ID NO:4) has an identical amino acid sequence to full-length Fas except that 21 amino acid residues, corresponding to the last 5 residues of the Fas extracellular domain and the first 16 amino acid residues of the Fas hydrophobic transmembrane domain, are missing. Thus, this product could be expressed as a soluble, secreted form of Fas.

FasΔ2 (SEQ ID NO:5) has a 109-bp deletion derived from nucleotide sequences 529-GC TTA GAA to TGC ACC AA-637, that gives rise to an out of frame amino acid sequence. A second 83-bp deletion was present from nucleotide sequences 763-TG AAG AGA to TTA AAT CCT-845. As noted, the nucleotide 763 is positioned immediately after the nucleotide deletion observed in the FasΔ1. The novel polypeptide that this transcript encodes (SEQ ID NO:6), consists of 149 amino acid residues with a calculated molecular weight of 16,646.

FasΔ3 (SEQ ID NO:7) has a 247-bp deletion starting at nucleotide sequence 391-GT GAA AGG and ending at nucleotide sequence TGC ACC AA-637. This deletion also causes frame shifting, and the shifted frame gives rise to an altered amino acid sequence. It is capable of encoding a polypeptide of 103 amino acid residues (SEQ ID NO:8), with a calculated molecular weight of 11,434.

FasΔ4 (SEQ ID NO:9) has a 310-bp deletion derived from the regions described in FasΔ1 and FasΔ3. It is possible that this variant is generated from a *fas* mRNA transcript which has undergone alternative splicing

identical to that observed in FasΔ1 and FasΔ3 (FIG. 3). If the FasΔ4 is capable of encoding a protein product, it would represent only 86 amino acid residues (SEQ ID NO:10) and have a calculated molecular weight of 9,389.

Hydropathy plot analysis (Gavrieli *et al.*, 1992) revealed that the putative polypeptides encoded by FasΔ2, FasΔ3 and FasΔ4 retain the hydrophobic leader peptide of the previously reported normal Fas antigen, but do not have the hydrophobic transmembrane domain in their altered amino acid sequences. The absence of the transmembrane domain suggests that they might also be expressed as soluble forms and may be secreted.

EXAMPLE II

Soluble Fas as an Activity Marker for SLE and AILD

As stated in Example I, abnormally-sized *fas* transcripts were found in SLE and AILD patients using RT-PCR™. Although normal controls and patients exhibited transcripts of the same sizes (FIG. 1), it is important to note that, in some patients, the relative intensity of the full-length *fas* mRNA was decreased, whereas the intensity of the FasΔ1 mRNA was increased. Diagnostic tests using any two appropriate *fas* primers in PCR™ may thus be conducted to identify SLE and AILD patients, without the need for any novel primers.

Now that the area of the deletion in the smaller transcripts, and the contiguous sequence spanning the breakpoint in the mutants, has been defined, this opens the way for soluble Fas detection *via* PCR™. This would impart a particular practical utility to short *fas* nucleic acid probes of the present invention, as they would be useful in diagnostic screening embodiments to identify certain SLE and AILD patients. In addition to

the teachings of the previous examples, the techniques of PCR™ are generally well known to those of skill in the art and are described in numerous publications, including U.S. Patent 4,603,102, which is incorporated herein by reference for the purposes of teaching how to conduct PCR™.

The question of whether Fas expression in any given cell or patient is represented by full-length Fas expression, or by expression of the Fas exon deletion variant, FasΔ1, can also be assessed using solution hybridization/RNase protection, as described in Example VI.

EXAMPLE III
Identification of Soluble Human Fas Proteins

A. Methods

1. **Fas-Expression Vectors**. The full-length *fas* cDNA and the FasΔ1 variant that encodes a novel soluble form of Fas antigen were excised from the subclones, *fas* cDNA and FasΔ1, using the *Eco*RI restriction enzyme, and purified from low melting temperature agarose. The fragments were inserted into the *Eco*RI cloning site of the expression vector pcDNA I (Invitrogen Co., San Diego, CA). The orientations of the inserts with respect to the human CMV enhancer/promoter sequences were determined by *Hind* III digestion and agarose gel electrophoresis. The Fas expression recombinants were then purified with QIAGEN 100-tips.

Twenty micrograms of purified DNA was transfected into 5×10^6 COS-7 cells by DEAE-dextran-mediated DNA transfection with the addition of chloroquine treatment (Kaufman and Sharp, 1982). Approximately 5×10^6 COS-cells were cultured in 10 cm petri dishes (Falcon, Lincoln

Park, NJ) in RPMI 1640 (Flow Laboratories, McLean, VA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin in 5% CO_2 at 37°C for 18 h. Then the medium was removed and replaced with 10 ml of RPMI 1640 containing 10 mM chloroquine (Sigma). Twenty micrograms of the recombinant plasmid was precipitated in 0.5 ml of the same medium containing 500 μg/ml DEAE-Dextran (Sigma), and added to the cells, which were then cultured in 5% CO_2 at 37°C for 4 hours. The transfected cells were then treated with 10% DMSO in RPMI 1640 at room temperature for 2 minutes and the medium was replaced with fresh complete medium.

2. **Immunofluorescence and Flow Cytometry Analyses**. The COS-7 cells were detached from the dish after 72 hours of culture by replacing the medium with Hank's Buffer containing 1 mM EDTA at 37°C for 5 minutes. For surface staining, single cell suspensions of Fas-expression plasmid transfected COS cells (10^6/sample) were stained in PBS containing 5% FCS, 0.1% sodium azide, and 20 μg/ml of the anti-human Fas MAb (IgM) in PBS containing 0.5% (v/v) Tween-20® at room temperature for 30 minutes. After washing the cells three times with PBS, the cells were stained with FITC-conjugated goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL) for 30 minutes. As a control, the same cells were similarly treated, except that the primary antibody was replaced with an irrelevant mouse IgM. Viable cells (10,000 per sample) were analyzed by flow cytometry on a FACS-Scan (Becton-Dickinson, Mt. View, CA) with logarithmic scales.

Samples of these cell preparations also were centrifuged onto slides by using cytocentrifugation, mounted using 90% glycerol/10% PBS mounting fluid containing 0.01% p-phenyl nediamine (Sigma), and examined under a Nikon optiphot fluorescence microscope.

Photographs were taken using Kodak Ektachrome 800/1600 film and automatic exposure.

3. **Assay for Apoptosis Induced by Anti-Fas Ab.** Normal human PBMC were isolated as described and cultured in 5% CO^2 at 37°C in RPMI 1640 supplemented with 10% FCS, 2mM glutamine, 100 U/ml penicillin and 10 μg/ml streptomycin. The cells were stimulated with PHA-P (10 ug/ml) (Pharmacia, Piscataway, NJ) for 48 hours. Approximately $5x10^5$ stimulated cells were then cultured in a flat-bottom 96 well plate (Costar Corp., Cambridge, MA) in 150 μl RPMI 1640 supplemented with 10% FCS and 200 ug/ml murine anti-human Fas MAb (IgM) in the presence of serial dilutions of supernatants derived from the COS-7 cells transfected with either Fas cDNA-or FasΔ1-expression plasmid. The supernatants were collected after culture for 3 days to permit transient expression of the inserted sequences in the mammalian expression vector.

After 12 hours incubation, approximately $2x10^5$ of the cells were cytospun onto poly-L-lysine pretreated slides, fixed in 10% (v/v) formalin at room temperature for 12 hours, and then *in situ* apoptosis staining was performed as previously described (Wylliams *et al.*, 1990). The slide was rinsed with dH_2O and incubated with 20 ug/ml proteinase K (Sigma) for 15 min at room temperature. The slide was then washed thoroughly with dH_2O 3 times and immersed in terminal deoxynucleotide transferase (TdT) reaction mix containing terminal TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride), TdT (0.5 units/ml) and digitonigen modified dUTP (0.5 μmol) in a humid atmosphere at 37°C for 60 minutes. The reaction was terminated by transferring the slide to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 minutes at room temperature. The slide was blocked with 10% FCS in PBS for 30 minutes at room temperature and then incubated

with alkaline phosphatase (AP)-conjugated anti-digitonigen Ab at 1:50 dilution in 10% FCS in PBS for 60 minutes at room temperature, washed in PBS 3 times, and developed with AP substrate for 30 minutes at room temperature. A minimum of 200 lymphocytes were counted in 10 randomly selected microscopic fields by an observer unaware of the cell culture conditions and the percentage of cells undergoing apoptosis was determined.

B. Results

To confirm the existence of the soluble Fas products, the full-length *fas* cDNA (SEQ ID NO:1), and FasΔ1 (SEQ ID NO:3), were expressed, using a mammalian expression vector and COS cells, and then flow cytometry and immunohistochemical analysis were used to stain the transfected COS cells using an anti-human Fas Ab. FasΔ1 was selected for initial studies as it most likely encodes a polypeptide that is present in soluble form, is secreted and, most significantly, retains the conformation of the Fas extracellular domain to which the anti-Fas antibody binds. The full-length *fas* cDNA was used as a control.

The *fas* cDNA and FasΔ1 clones were inserted into a mammalian expression vector (pc DNA I) and transfected into COS cells. RT-PCR™ analysis indicated that the transfected cells expressed the corresponding *fas* transcript. Immunofluorescence and flow cytometry analysis revealed that the antibody stained the surface of the cells that were transfected with the full-length *fas* cDNA, but not those cells transfected with FasΔ1. It also revealed that there were no apparent difference in the degree of cytoplasmic staining between those cells transfected with the full-length *fas* cDNA and those cells transfected with the FasΔ1 variant.

Taken together, these data confirmed that the FasΔ1 transcript produced the expected Fas protein product as did the *fas* cDNA, and in addition, suggested that FasΔ1 protein was not retained in the membrane of the cell, but was present in the cytoplasm as a soluble form. The presence of the soluble form of the FasΔ1 antigen in the cytoplasm of the transfected COS cells was also confirmed by biosynthetic labeling of the proteins with ^{35}S-methionine, immunoprecipitation and SDS-PAGE electrophoresis.

The presence of a secreted FasΔ1 protein was confirmed by an inhibition assay for apoptosis induced by the anti-Fas Ab. Phytohemagglutinin (PHA-P)-stimulated PBMC from normal subjects were used for this study as it has been shown that stimulated cells undergo apoptosis after culture with the anti-Fas Ab (Owen-Schaub *et al.*, 1992). The PHA-P-stimulated PBMC were cultured with anti-Fas Ab in the presence of supernatant derived from COS cells transiently expressing FasΔ1 antigen from the FasΔ1-expression recombinant. As a control, the same PBMC cells were similarly treated with the anti-human Fas Ab except that the added supernatant was derived from COS cells transiently expressing Fas antigen from the full-length Fas cDNA-expression recombinant. The percentage of cells undergoing apoptosis was determined 12 h later by the TdT method.

There was a dose-dependent increase in cell survival rate in the medium supplemented with the supernatant from COS cells transfected with the FasΔ1-expression plasmid but not in the medium supplemented with the supernatant from COS cells transfected with the full-length Fas cDNA-expression plasmid (FIG. 4). This higher survival rate observed in cells treated with the FasΔ1 supernatant can be explained by the secretion of the soluble FasΔ1 antigen into the supernatant, resulting in interaction of

the soluble FasΔ1 antigen with anti-human Fas Ab, neutralization of the Ab and consequently, inhibition of the Ab-mediated apoptosis.

EXAMPLE IV

Cloning of a *fas* Gene Deletion Mutant from Human Placenta

A. Methods

The isolation of PBMC, isolation of RNA, northern blot analysis, cDNA probe, oligonucleotide synthesis, reverse transcription PCR™, PCR™ product cloning and nucleotide sequencing and analysis were performed as described in Example I.

1. **Human *fas* Gene Cloning**. Human placenta genomic DNA library in the Lambda FIX™II Vector (Stratagene Inc., La Jolla, CA) was screened by plaque hybridization under high stringency conditions (Sambrook *et al.*, 1989) using [α-^{32}P]-dCTP labeled *fas* cDNA probes, of which one had *fas* nucleotides 170-1336 (SEQ ID NO:1), and another had *fas* nucleotides 1316 to 2441 SEQ ID NO:16). The probes were labeled by using the random hexanucleotide priming method (Feinberg and Vogelstein, 1984). The positive clones were isolated and the recombinant λ DNA were prepared using QIAGEN 100-tips (QIAGEN Inc., Studio, CA) following the procedure recommended by the supplier. The appropriate DNA fragments of the inserts in the vector were subcloned into the Bluescript vector (Stratagene).

2. **Ribonuclease (RNase) Protection Assays**. The *fas* cDNA corresponding to nucleotide 565 to 767 was amplified by using 20 ng plasmid DNA of the cloned full-length *fas* cDNA in pCR™ vector and primers, 5'-*GGAATTC*ATACCAAGTGCAGATGGTA-3' (SEQ ID NO:13; including nucleotides 565 to 582) and 5'-*ATCAAGCTT*CACCCAAACAATTAGTGG-3' (SEQ ID NO:14;

complementary to nucleotides 747 to 767). Underlined nucleotide represent *EcoR*I and *Hind*III restriction sites in the sense and antisense primers, respectively. *Italics* are used to indicate nucleotide not derived from the *fas* cDNA sequence.

Amplification was carried out for 30 cycles. Each cycle consisted of 60 s denaturation at 94°C, 90 s annealing at 50°C, and 60 s extension at 72°C. The amplified *fas* cDNA was purified after agarose gel electrophoresis, digested with *EcoR*I and *Hind*III, and subcloned into pGEM-7Zf (+). Transcription reactions were carried out by using 1 μg *EcoR*I linearized DNA template, 50 mCi [α-^{32}P]-CTP (Amersham Corp., Arlington Heights, IL) and 20 units SP6 RNA polymerase (transcription *in vitro* systems; Promega), according to the instructions of the supplier. The reaction was terminated by the addition of 2 units RQ1 RNase-free DNase (Promega). The radiolabeled antisense RNA probe was 258 nucleotide in size, of which the 203 nucleotide was complementary to *fas* mRNA at nucleotide 565 to 767 and 55 nucleotides were complementary to the vector sequence between the SP6 transcription initiation start site and the *Hind* III cloning site (SEQ ID NO:15). This probe is described more fully in Example VI.

RNase protection studies were performed by using the RPA II kit (Ambion, Austin, TX), according to the instructions of the supplier. Briefly, approximately 8×10^4 cpm of antisense RNA probe was ethanol-precipitated with 50 μg cellular RNA from PBMC. The resulting pellets were dried and resuspended in 20 μl of hybridization buffer containing 80% formamide. The mixture was heated at 90°C for 3 min and then incubated for 16 h at 42°C. RNase digestion was performed by adding 200 μl of a 1/100 dilution of a stock solution containing 250 units/ml of RNase A and 10,000 units/ml of RNase T1 and incubating at

37°C for 30 min. RNase-resistant fragments were ethanol-precipitated, resuspended in 8 μl of loading dye containing 80% formamide, electrophoresed on a 6% polyacrylamide, 7 M urea gel and visualized by autoradiography for 36 h at -70°C. Negative controls included in each study consisted of mixtures of antisense RNA probe and 10 μg yeast tRNA.

B. Results

To elucidate the basis of the FasΔ1 (also designated FasΔTM) variant at the gene level, the *fas* gene was cloned from a human placenta genomic DNA library. Genomic DNA sequencing of the human *fas* gene revealed that FasΔ1, with the 63 nucleotide deletion, corresponded to an intact exon and therefore, suggested this mRNA variant was most likely due to splicing out of the exon (Smith *et al.*, 1989).

The DNA sequence analysis also revealed a 152 bp intron and a 1183 bp intron flanking the TM-encoding exon. Splicing of the intron donor site to the next intron splice acceptor would accurately yield the FasΔ1 (FasΔTM) transcript.

To confirm that FasΔ1 (FasΔTM) detected by RT-PCR™ in the PBMC was present in the cell and not due to a RT-PCR™ artifact, a solution hybridization and RNase protection study was carried out. The radiolabeled antisense RNA probe was 258 nucleotide in size, of which the 203 nucleotide was complementary to *fas* mRNA at nucleotide 565 to 767 and 55 nucleotides were complementary to the vector sequence between the SP6 transcription initiation start site and the *Hind* III cloning site (SEQ ID NO.15).

The probe hybridized with PBMC cellular RNA, digested with RNase A and T1, and electrophoresed. Autoradiography revealed two PBMC mRNA protected fragments which were 203 and 135 nucleotides in length. As indicated in the interpretative diagram of FIG. 5, the 203 and 135 nucleotide long fragments were protected by *fas* mRNA species with no deletion or with the deletion of a 63 nucleotide intact exon, respectively. The ratio of the intensity of these protected bands was in agreement with the abundance of RT-PCR™ products that represented full-length *fas* cDNA and FasΔ1 (FasΔTM).

EXAMPLE V

Biological Activity of Soluble Fas

To demonstrate that soluble Fas is biologically active, mouse-soluble Fas was produced to determine the role of this molecule *in vivo*. The soluble Fas was injected (2.5 μg per gram of body weight intraperitoneally) every day for 3 days. This treatment gave rise to an average serum concentration comparable to that observed in the SLE patients described in Example IX. There was an increase in $CD4^-CD8^-$ as well as $CD4^+$ and $CD8^+$ (single positive) thymocytes with a decrease in $CD4^+CD8^+$ (double positive) thymocytes. There was a threefold increase in the number of spleen cells, primarily due to an increase in B cells.

These data demonstrate that levels of soluble Fas observed in SLE patients can have a dramatic effect on lymphocyte development *in vivo*. These alterations in phenotypic development resulted in increased autoreactivity, as evidenced by a fourfold increase in the response of syngenetic, mixed cell culture proliferation by thymocytes and spleen cells from soluble Fas-treated mice, compared to those from control-treated mice. These results demonstrate that soluble Fas *in vivo*

can lead to altered lymphocyte development and increased proliferation in response to self antigens.

EXAMPLE VI

Antisense Probes to Detect Soluble *fas* mRNA

The availability of radiolabeled antisense *fas* RNA probes would be an advantage, allowing one to determine whether Fas expression in any given cell is represented by full-length Fas expression or by expression of the Fas exon deletion variant FasΔ1 (FasΔTM) that encodes a transmembrane deletion mutant.

To achieve this, a *fas* cDNA fragment spanning the transmembrane exon was inserted into a pGEM-7Z vector. The radiolabeled antisense RNA probe was 258 nucleotide in size, of which the 203 nucleotide was complementary to *fas* mRNA at nucleotide 565 to 767 and 55 nucleotides were complementary to the vector sequence between the SP6 transcription initiation start site and the *Hind* III cloning site (SEQ ID NO.15). This probe was used in solution hybridization/RNase protection to detect *fas* mRNAs.

RNase protection studies were performed by using the RPA II kit (Ambion, Austin, TX), according to the instructions of the supplier. Briefly, approximately 8×10^4 cpm of antisense RNA probe was ethanol-precipitated with 50 μg cellular RNA from PBMC. The resulting pellets were dried and resuspended in 20 μl of hybridization buffer containing 80% formamide. The mixture was heated at 90°C for 3 min and then incubated for 16 h at 42°C. RNase digestion was performed by adding 200 μl of a 1/100 dilution of a stock solution containing 250 units/ml of RNase A and 10,000 units/ml of RNase T1 and incubating at 37°C for 30 min. RNase-resistant fragments were ethanol-precipitated, resuspended in 8 μl of loading dye

containing 80% formamide, electrophoresed on a 6% polyacrylamide, 7 M urea gel and visualized by autoradiography for 36 h at -70°C. Negative controls included in each study consisted of mixtures of antisense RNA probe and 10 μg yeast tRNA.

EXAMPLE VII

Monoclonal Antibodies (MAbs) to Soluble Fas

1. Monoclonal Antibodies Specific for the Mouse Fas Antigen

The inventors have generated three anti-mouse Fas antibodies in different species (C57BL/6-*lpr/lpr* mice, rat, guinea pig) and have polyclonal and monoclonal sera from each species.

In one instance, Fas expression defective C57BL/6-*lpr* female mice were immunized with the murine Fas-hIgG1 fusion protein (Example XIII) and fused the immune spleen cells with AGx8 cells. On ELISA analysis the selected MAb (clone 2A36:murine IgG1) bound to the Fas-hIgG1 fusion protein, but not to the control CTLA4-hIgG1 fusion protein. MAb 2A36 also bound to the surface of COS cells transfected with the full-length murine *fas* cDNA, but not to cells transfected with the control vector.

Significantly, MAb 2A36 was found to induce apoptosis in *fas* cDNA transfected COS cells. 10^6 COS cells were transfected with the pcDNA1 expression vector containing the full-length *fas* cDNA. After 48 hr, the cells were incubated with either a control antibody or the anti-Fas MAb (approx. 1mg/ml). The viability of the cells was assessed 12 hr later by phase contrast microscopy. Similarly treated cells were stained with Trypan Blue (0.25% in PBS) and immediately assessed for

viability. Five randomly selected fields were counted and over 200 cells counted per field. The value indicated in the histogram represent the mean ± the standard error of the mean.

In addition to MAb 2A36, each of the other monoclonal antibodies also detected the Fas protein on the surface of lymphocytes from MRL-+/+ mice and, at lower levels, on MRL-*lpr/lpr* mice. These antibodies immunoprecipitate a 45 kD protein from lymphocytes from MRL-+/+ and a 45kD and 60kD protein from *lpr/lpr* mice. The mouse and rat anti-mouse Fas antibodies detect high expression of Fas in the cytoplasm of liver cells and on the surface of IFN-γ activated T cells.

2. Monoclonal Antibodies Specific for the Human Fas Antigen

A MAb to human Fas antigen has been prepared by immunization of female SD rats with COS-7 cells transfected with the full-length human *fas* cDNA. The immunized cells were fused with Agx8 cells and screened by FACS analysis of Fas^+ CEM cells, using Fas^- CEM cells as the negative controls. This MAb induces apoptosis, as shown by apoptosis of CEM-6 cells, induction of DNA degradation in Fas^+ cells and *in situ* apoptosis staining. The MAb also reacts with a protein of 50 kDa on the basis of western blot analysis.

EXAMPLE VIII

ELISA to Detect Soluble Fas

In a particular embodiment of a soluble Fas ELISA assay, a 96 well microtiter plate is coated with Anti-Fas MAb and additional protein binding sites are blocked by BSA in coupling buffer. The expressed and purified Fas antigen is used for establishing a standard curve.

Control samples may also include COS cells transfected with a full-length human Fas expression clone.

Different wells of the microtiter plate coated with Anti-Fas MAb are contacted with the samples containing known amounts of soluble Fas (standards) and with biological samples in which the soluble Fas levels are unknown. After sufficient time for binding, the wells are extensively washed with PBS containing 0.1% Tween-20® to remove unbound antibodies and other proteins from the wells. Then 100 μl of a diluted second anti-Fas mAb derived from another species is added and allowed to bind, followed by 100 μl of 1:1000 dilution of alkaline phosphatase conjugated (tertiary) antibody in PBS containing 3% BSA and 0.1% Tween-20®. The reaction is developed with 100 μl of substrate solution and the optical density (OD) is read at 405 nm on a Titertek multiscanner. The absorbance value for each tested serum is derived from the mean value of duplicated wells after subtracting the background absorbency value obtained against BSA.

EXAMPLE IX

Detection of Soluble Fas in Autoimmune Disease by ELISA

Sera from systemic lupus erythematosus (SLE) and Rheumatoid Arthritis (RA) patients and healthy unrelated control subjects were collected and tested using a version of the above ELISA method. Plates were coated with anti-human Fas MAb (1 μg/ml) blocked with 1% BSA. 100 μl of the sera (1:10, 1:40, 1:50, 1:250 diluted in 1% BSA or in PBS containing 0.1% Tween-20®) was added in duplicate to microtiter plate wells. Polyclonal rat anti-human Fas sera, diluted 1:100 in 1% BSA was used to detect the bound human Fas, and this was detected using an anti-rat IgG alkaline phosphatase antibody.

Control samples included Cos cells transfected with a full-length human Fas expression clone, or a human Fas fusion protein.Values > mean + 2 s.d. obtained from the healthy individuals were considered positive. Statistical analysis was performed using the student's t-test and chi-square. In these studies, it was found that control supernate and normal sera contained low levels of soluble human Fas (FIG. 6). High levels of soluble Fas were also detected in supernatant of transfected COS cells.

Importantly, it was found that human subjects with SLE had elevated sera levels of soluble Fas (FIG. 6). A soluble form of Fas was present at increased concentrations in about 60% of patients with SLE. In normal controls and patients with rheumatoid arthritis, serum levels of soluble Fas were less than 100 ng/ml. Although soluble Fas is only increased approximately twofold in 60% of SLE patients, much higher physiological concentrations may be present at limited sites where there is high expression of Fas and Fas ligand in certain lymphoid organs,

The results in FIG. 6 show that this ELISA assay can be used to detect soluble Fas in the serum of human patients and, thereby, to diagnose certain patients with autoimmune disease. It is possible that other disorders, such as ankylosing spondylitis and vasculitis, may be found to be characterized by increased levels of soluble Fas. Indeed, it has been determined that some patients with Rheumatoid Arthritis exhibited elevated levels of soluble Fas.

It should be emphasized that diagnostic ELISAs such as these provide a definite utility for the soluble Fas antigen of the present invention as this antigen may be

employed to establish the standard curve necessary for the "calibration" of such ELISAs.

EXAMPLE X

Defective Cellular Fas Antigen in Autoimmunity

This example shows the defective Fas Antigen expression detected by the Fas MAb (2A36) on unstimulated and IFN-γ stimulated lymphocytes from *lpr/lpr* mice, a widely accepted animal model of autoimmunity.

Flow cytometry analysis using the 2A36 MAb and freshly isolated lymph node (LN) cells indicated decreased expression of the Fas antigen in MRL-*lpr* mice compared to +/+ mice. Fas expression was analyzed on LN cells isolated from MRL-+/+ and -*lpr* mice after culture with 200 u/ml of rIFN-γ for 48 hr. After stimulation, there was a distinct peak of Fas^{bright} LN cells from +/+, and up-regulation of Fas expression on LN cells from *lpr* mice. The Fas^{bright} lymphocytes from the +/+ mice were small cells that appeared to be undergoing apoptosis as determined by fluorescence and light microscopy. These results suggest that there is a defect in up-regulation of Fas expression after IFN-γ stimulation of lymphocytes from *lpr* mice, and a corresponding defect in apoptosis.

Apoptosis was also determined by propidium iodide staining (PI). After IFN-γ stimulation, almost all of the Fas$^+$ cells from the +/+ mice were non-viable as determined by PI staining, compared to 50% non-viable cells from *lpr* mice. These data indicate that Fas expression is highly correlated with cell death in +/+ mice, but less so in *lpr* mice. As *lpr* lymphocytes express low or abnormal Fas, 50% of the cell death after IFN-γ stimulation may occur through a Fas independent mechanism. The death of almost all Fas$^+$ cells in lpr mice, compared to death of only 50% of Fas$^-$ cells in +/+

mice, suggests that Fas expression aids in cell death during culture, possibly by interacting with the Fas-ligand that has been proposed to be upregulated after IFN-γ stimulation.

EXAMPLE XI

Soluble Fas as an Inhibitor of Apoptosis

To determine whether soluble Fas inhibits apoptosis of PBMC, or other Fas$^+$ cells, in humans, sera from patients with high titer of soluble Fas are incubated at different concentrations with PHA-P stimulated normal human PBMC as described in Example III. PHA-P stimulated human PBMC undergo a Fas dependent apoptosis, presumably from interaction with the Fas-ligand secreted in an autocrine or paracrine fashion. Naturally occurring soluble Fas present at physiological concentrations in the sera of patients would bind to the Fas-ligand and inhibit Fas-Fas-ligand mediated apoptosis.

The finding of a circulating inhibitor of apoptosis in patients with autoimmune disease would be highly significant since it would then be possible to down-modulate or clear soluble Fas by plasmapheresis, thereby restoring normal apoptosis and reversing certain autoimmune diseases.

EXAMPLE XII

Fas Allows T cell Proliferation

To determine whether defective Fas expression allows excessive proliferation of T cells, 5x10^5 total spleen cells from MRL-+/+ and -*lpr/lpr* mice were cultured with 5 μg/ml of precoated anti-CD3 antibody in the absence or presence of 5 μg/ml of purified Fas-Ig fusion protein. The proliferation was determined by ^{3}H thymidine incorporation at various times after culture.

It was found that the proliferative response was increased and prolonged by addition of Fas-Ig fusion protein to cultures of +/+ spleen cells. In contrast, Fas-Ig fusion protein had no effect on the culture of CD3 stimulated spleen cells from *lpr* mice. These results suggest that: 1) The Fas-Ig fusion protein allows prolonged survival and proliferation of T cells after stimulation; 2) The Fas-Ig fusion protein neutralizes or binds to Fas-ligand in these culture systems; 3) Fas-Fas-ligand interaction play a key role in selection of cells destined to survive or undergo apoptosis after CD3 signaling.

This data suggests that since soluble Fas inhibits apoptosis after activation, soluble Fas could be used to maintain growth of T cells or other cells susceptible to Fas apoptosis. Such maintenance could be *in vivo*, or it could be *in vitro*, *e.g.*, in the maintenance of T cells. This imparts yet another practical usefulness to the present invention as the culture and maintenance of T cells is known to be technically difficult.

EXAMPLE XIII

Cloning the Fas Ligand

Creation of a Fas/hIgG1 Fusion Protein, a Cloning Tool

Expression and cloning of the Fas-ligand was accomplished using the extracellular domain of a Fas antigen (Fas-ED)/hIgG1 fusion protein as a specific probe to identify and screen a spleen cDNA library to obtain the Fas-ligand.

A fas/hIgG1 fusion gene was constructed and transfected into COS cells. The full-length mouse Fas antigen was 306 amino acids (aa) long, with a calculated M_r of ~35 kD. It contained a single transmembrane

domain, an extracellular domain of 148aa and a cytoplasmic domain. The M_r on SDS gel electrophoresis was approximately 10 kD larger due to glycosylation. Human IgG1 is a soluble homodimer, the constant regions of which are divided into CH1, CH2 and CH3 domains with a flexible hinge region between CH1 and CH2.

The fusion protein was constructed with the 148 aa-long Fas extracellular domain at its N-terminus and the 247 aa-long hinge, CH2 and CH3 regions of human IgG1 at its C-terminus. The two peptides were connected by an 11aa polylinker sequence from the vector. The fusion protein was used in preference to the full-length Fas antigen for screening as the fusion protein is a secretory Ig-like homodimer, and purification and assay of the fusion protein is readily accomplished by using anti-human IgG secondary reagents.

A 530 bp sequence (26bp~556bp) containing the extracellular sequence of the *fas* gene was PCR™ amplified from a mouse *fas* cDNA clone. The 5′ primer used was linked to a HindIII linker and the 3′ primer linked to a SalI linker. The PCR™ product was then ligated into pG1-CH2 plasmid (obtained from Dr. T.F. Tedder) which originated from a pSP65 vector with an insert of the hinge, CH2 and CH3 fragment of human IgG1 at the HindIII and SalI sites to form an in-frame junction with the hIgG fragment. The fusion gene was sequenced to confirm that *fas* extracellular domain was in-frame with the hIgG fragment.

The fusion gene was then excised using EcoR1 and HindIII, ligated into a pcDNAI eukaryotic expression vector and transfected into the COS 7 cell line by the DEAE-dextran method. The fusion gene was expressed from the CMV/T7 promoter and the protein was secreted into the culture medium and screened by an anti-hIgG ELISA. The

fusion protein was precipitated from the supernatant of the transfected Cos cells by anti-hIgG agarose. After precipitation and electrophoresis, the Fas/hIgG fusion protein was characterized by western blot assay using anti-hIgG as the probe. The prominent new protein band observed at approximately 67.5 kD corresponded to the predicted molecular weight of the Fas/hIgG fusion protein.

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: UAB RESEARCH FOUNDATION
(B) STREET: 701 South 20th Street, Suite 1120G
(C) CITY: Birmingham
(D) STATE: Alabama
(E) COUNTRY: United States of America
(F) POSTAL (ZIP) CODE: 35294-0111

(ii) TITLE OF INVENTION: SECRETED HUMAN FAS ANTIGEN

(iii) NUMBER OF SEQUENCES: 16

(iv) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: UNKNOWN
(B) FILING DATE: CONCURRENTLY HEREWITH
(C) CLASSIFICATION: UNKNOWN

(v) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: USSN 08/371,263
(B) FILING DATE: 23-DEC-1994

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1167 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CACTTCGGAG GATTGCTCAA CAACCATGCT GGGCATCTGG ACCCTCCTAC CTCTGGTTCT 60

TACGTCTGTT GCTAGATTAT CGTCCAAAAG TGTTAATGCC CAAGTGACTG ACATCAACTC 120

CAAGGGATTG GAATTGAGGA AGACTGTTAC TACAGTTGAG ACTCAGAACT TGGAAGGCCT 180

GCATCATGAT GGCCAATTCT GCCATAAGCC CTGTCCTCCA GGTGAAAGGA AAGCTAGGGA 240

CTGCACAGTC AATGGGGATG AACCAGACTG CGTGCCCTGC CAAGAAGGGA AGGAGTACAC 300

AGACAAAGCC CATTTTTCTT CCAAATGCAG AAGATGTAGA TTGTGTGATG AAGGACATGG 360

CTTAGAAGTG GAAATAAACT GCACCCGGAC CCAGAATACC AAGTGCAGAT GTAAACCAAA 420

CTTTTTTTGT AACTCTACTG TATGTGAACA CTGTGACCCT TGCACCAAAT GTGAACATGG 480

AATCATCAAG GAATGCACAC TCACCAGCAA CACCAAGTGC AAAGAGGAAG GATCCAGATC 540

TAACTTGGGG TGGCTTTGTC TTCTTCTTTT GCCAATTCCA CTAATTGTTT GGGTGAAGAG 600

AAAGGAAGTA CAGAAAACAT GCAGAAAGCA CAGAAAGGAA AACCAAGGTT CTCATGAATC 660

TCCAACCTTA AATCCTGAAA CAGTGGCAAT AAATTTATCT GATGTTGACT TGAGTAAATA 720

TATCACCACT ATTGCTGGAG TCATGACACT AAGTCAAGTT AAAGGCTTTG TTCGAAAGAA 780

TGGTGTCAAT GAAGCCAAAA TAGATGAGAT CAAGAATGAC AATGTCCAAG ACACAGCAGA 840

ACAGAAAGTT CAACTGCTTC GTAATTGGCA TCAACTTCAT GGAAAGAAAG AAGCGTATGA 900

CACATTGATT AAAGATCTCA AAAAAGCCAA TCTTTGTACT CTTGCAGAGA AAATTCAGAC 960

TATCATCCTC AAGGACATTA CTAGTGACTC AGAAAATTCA AACTTCAGAA ATGAAATCCA 1020

AAGCTTGGTC TAGAGTGAAA AACAACAAAT TCAGTTCTGA GTATATGCAA TTAGTGTTTG 1080

AAAAGATTCT TAATAGCTGG CTGTAAATAC TGCTTGGTTT TTTACTGGGT ACATTTTATC 1140

ATTTATTAGC GCTGAAGAGC CAACATA 1167

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 335 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala
1               5                   10                  15

Arg Leu Ser Ser Lys Ser Val Asn Ala Gln Val Thr Asp Ile Asn Ser
            20                  25                  30

Lys Gly Leu Glu Leu Arg Lys Thr Val Thr Thr Val Glu Thr Gln Asn
        35                  40                  45

Leu Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro
    50                  55                  60

Pro Gly Glu Arg Lys Ala Arg Asp Cys Thr Val Asn Gly Asp Glu Pro
65                  70                  75                  80

Asp Cys Val Pro Cys Gln Glu Gly Lys Glu Tyr Thr Asp Lys Ala His
                85                  90                  95

Phe Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Glu Gly His Gly
            100                 105                 110
```

```
Leu Glu Val Glu Ile Asn Cys Thr Arg Thr Gln Asn Thr Lys Cys Arg
        115                 120                 125

Cys Lys Pro Asn Phe Phe Cys Asn Ser Thr Val Cys Glu His Cys Asp
    130                 135                 140

Pro Cys Thr Lys Cys Glu His Gly Ile Ile Lys Glu Cys Thr Leu Thr
145                 150                 155                 160

Ser Asn Thr Lys Cys Lys Glu Glu Gly Ser Arg Ser Asn Leu Gly Trp
                165                 170                 175

Leu Cys Leu Leu Leu Leu Pro Ile Pro Leu Ile Val Trp Val Lys Arg
            180                 185                 190

Lys Glu Val Gln Lys Thr Cys Arg Lys His Arg Lys Glu Asn Gln Gly
        195                 200                 205

Ser His Glu Ser Pro Thr Leu Asn Pro Glu Thr Val Ala Ile Asn Leu
    210                 215                 220

Ser Asp Val Asp Leu Ser Lys Tyr Ile Thr Thr Ile Ala Gly Val Met
225                 230                 235                 240
```

```
Thr Leu Ser Gln Val Lys Gly Phe Val Arg Lys Asn Gly Val Asn Glu
                245                 250                 255

Ala Lys Ile Asp Glu Ile Lys Asn Asp Asn Val Gln Asp Thr Ala Glu
            260                 265                 270

Gln Lys Val Gln Leu Leu Arg Asn Trp His Gln Leu His Gly Lys Lys
        275                 280                 285

Glu Ala Tyr Asp Thr Leu Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys
    290                 295                 300

Thr Leu Ala Glu Lys Ile Gln Thr Ile Ile Leu Lys Asp Ile Thr Ser
305                 310                 315                 320

Asp Ser Glu Asn Ser Asn Phe Arg Asn Glu Ile Gln Ser Leu Val
                325                 330                 335
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1104 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
CACTTCGGAG GATTGCTCAA CAACCATGCT GGGCATCTGG ACCCTCCTAC CTCTGGTTCT    60
TACGTCTGTT GCTAGATTAT CGTCCAAAAG TGTTAATGCC CAAGTGACTG ACATCAACTC   120
CAAGGGATTG GAATTGAGGA AGACTGTTAC TACAGTTGAG ACTCAGAACT TGGAAGGCCT   180
GCATCATGAT GGCCAATTCT GCCATAAGCC CTGTCCTCCA GGTGAAAGGA AAGCTAGGGA   240
CTGCACAGTC AATGGGGATG AACCAGACTG CGTGCCCTGC CAAGAAGGGA AGGAGTACAC   300
AGACAAAGCC CATTTTTCTT CCAAATGCAG AAGATGTAGA TTGTGTGATG AAGGACATGG   360
CTTAGAAGTG GAAATAAACT GCACCCGGAC CCAGAATACC AAGTGCAGAT GTAAACCAAA   420
CTTTTTTTGT AACTCTACTG TATGTGAACA CTGTGACCCT TGCACCAAAT GTGAACATGG   480
AATCATCAAG GAATGCACAC TCACCAGCAA CACCAAGTGC AAAGAGGAAG TGAAGAGAAA   540
```

GGAAGTACAG AAAACATGCA GAAAGCACAG AAAGGAAAAC CAAGGTTCTC ATGAATCTCC 600

AACCTTAAAT CCTGAAACAG TGGCAATAAA TTTATCTGAT GTTGACTTGA GTAAATATAT 660

CACCACTATT GCTGGAGTCA TGACACTAAG TCAAGTTAAA GGCTTTGTTC GAAAGAATGG 720

TGTCAATGAA GCCAAAATAG ATGAGATCAA GAATGACAAT GTCCAAGACA CAGCAGAACA 780

GAAAGTTCAA CTGCTTCGTA ATTGGCATCA ACTTCATGGA AAGAAAGAAG CGTATGACAC 840

ATTGATTAAA GATCTCAAAA AAGCCAATCT TTGTACTCTT GCAGAGAAAA TTCAGACTAT 900

CATCCTCAAG GACATTACTA GTGACTCAGA AAATTCAAAC TTCAGAAATG AAATCCAAAG 960

CTTGGTCTAG AGTGAAAAAC AACAAATTCA GTTCTGAGTA TATGCAATTA GTGTTTGAAA 1020

AGATTCTTAA TAGCTGGCTG TAAATACTGC TTGGTTTTTT ACTGGGTACA TTTTATCATT 1080

TATTAGCGCT GAAGAGCCAA CATA 1104

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 314 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala
1               5                   10                  15

Arg Leu Ser Ser Lys Ser Val Asn Ala Gln Val Thr Asp Ile Asn Ser
            20                  25                  30

Lys Gly Leu Glu Leu Arg Lys Thr Val Thr Thr Val Glu Thr Gln Asn
        35                  40                  45

Leu Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro
    50                  55                  60

Pro Gly Glu Arg Lys Ala Arg Asp Cys Thr Val Asn Gly Asp Glu Pro
65                  70                  75                  80
```

```
Asp Cys Val Pro Cys Gln Glu Gly Lys Glu Tyr Thr Asp Lys Ala His
                85                  90                  95

Phe Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Glu Gly His Gly
            100                 105                 110

Leu Glu Val Glu Ile Asn Cys Thr Arg Thr Gln Asn Thr Lys Cys Arg
        115                 120                 125

Cys Lys Pro Asn Phe Phe Cys Asn Ser Thr Val Cys Glu His Cys Asp
    130                 135                 140

Pro Cys Thr Lys Cys Glu His Gly Ile Ile Lys Glu Cys Thr Leu Thr
145                 150                 155                 160

Ser Asn Thr Lys Cys Lys Glu Glu Val Lys Arg Lys Glu Val Gln Lys
                165                 170                 175

Thr Cys Arg Lys His Arg Lys Glu Asn Gln Gly Ser His Glu Ser Pro
            180                 185                 190

Thr Leu Asn Pro Glu Thr Val Ala Ile Asn Leu Ser Asp Val Asp Leu
        195                 200                 205
```

```
Ser Lys Tyr Ile Thr Thr Ile Ala Gly Val Met Thr Leu Ser Gln Val
    210                 215                 220

Lys Gly Phe Val Arg Lys Asn Gly Val Asn Glu Ala Lys Ile Asp Glu
225                 230                 235                 240

Ile Lys Asn Asp Asn Val Gln Asp Thr Ala Glu Gln Lys Val Gln Leu
                245                 250                 255

Leu Arg Asn Trp His Gln Leu His Gly Lys Lys Glu Ala Tyr Asp Thr
            260                 265                 270

Leu Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys Thr Leu Ala Glu Lys
        275                 280                 285

Ile Gln Thr Ile Ile Leu Lys Asp Ile Thr Ser Asp Ser Glu Asn Ser
    290                 295                 300

Asn Phe Arg Asn Glu Ile Gln Ser Leu Val
305                 310
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 975 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
CACTTCGGAG GATTGCTCAA CAACCATGCT GGGCATCTGG ACCCTCCTAC CTCTGGTTCT     60

TACGTCTGTT GCTAGATTAT CGTCCAAAAG TGTTAATGCC CAAGTGACTG ACATCAACTC    120

CAAGGGATTG GAATTGAGGA AGACTGTTAC TACAGTTGAG ACTCAGAACT TGGAAGGCCT    180

GCATCATGAT GGCCAATTCT GCCATAAGCC CTGTCCTCCA GGTGAAAGGA AAGCTAGGGA    240

CTGCACAGTC AATGGGGATG AACCAGACTG CGTGCCCTGC CAAGAAGGGA AGGAGTACAC    300

AGACAAAGCC CATTTTTCTT CCAAATGCAG AAGATGTAGA TTGTGTGATG AAGGACATGA    360

TGTGAACATG GAATCATCAA GGAATGCACA CTCACCAGCA ACACCAAGTG CAAAGAGGAA    420

GGATCCAGAT CTAACTTGGG GTGGCTTTGT CTTCTTCTTT TGCCAATTCC ACTAATTGTT    480
```

```
TGGGGAAACA GTGGCAATAA ATTTATCTGA TGTTGACTTG AGTAAATATA TCACCACTAT     540
TGCTGGAGTC ATGACACTAA GTCAAGTTAA AGGCTTTGTT CGAAAGAATG GTGTCAATGA     600
AGCCAAAATA GATGAGATCA AGAATGACAA TGTCCAAGAC ACAGCAGAAC AGAAAGTTCA     660
ACTGCTTCGT AATTGGCATC AACTTCATGG AAAGAAAGAA GCGTATGACA CATTGATTAA     720
AGATCTCAAA AAAGCCAATC TTTGTACTCT TGCAGAGAAA ATTCAGACTA TCATCCTCAA     780
GGACATTACT AGTGACTCAG AAAATTCAAA CTTCAGAAAT GAAATCCAAA GCTTGGTCTA     840
GAGTGAAAAA CAACAAATTC AGTTCTGAGT ATATGCAATT AGTGTTTGAA AAGATTCTTA     900
ATAGCTGGCT GTAAATACTG CTTGGTTTTT TACTGGGTAC ATTTTATCAT TTATTAGCGC     960
TGAAGAGCCA ACATA                                                      975
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 149 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala
1               5                   10                  15

Arg Leu Ser Ser Lys Ser Val Asn Ala Gln Val Thr Asp Ile Asn Ser
            20                  25                  30

Lys Gly Leu Glu Leu Arg Lys Thr Val Thr Thr Val Glu Thr Gln Asn
        35                  40                  45

Leu Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro
    50                  55                  60

Pro Gly Glu Arg Lys Ala Arg Asp Cys Thr Val Asn Gly Asp Glu Pro
65                  70                  75                  80

Asp Cys Val Pro Cys Gln Glu Gly Lys Glu Tyr Thr Asp Lys Ala His
                85                  90                  95
```

```
Phe Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Glu Gly His Asp
            100                 105                 110

Val Asn Met Glu Ser Ser Arg Asn Ala His Ser Pro Ala Thr Pro Ser
        115                 120                 125

Ala Lys Arg Lys Asp Pro Asp Leu Thr Trp Gly Gly Phe Val Phe Phe
    130                 135                 140

Phe Cys Gln Phe His
145
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 920 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
CACTTCGGAG GATTGCTCAA CAACCATGCT GGGCATCTGG ACCCTCCTAC CTCTGGTTCT    60
TACGTCTGTT GCTAGATTAT CGTCCAAAAG TGTTAATGCC CAAGTGACTG ACATCAACTC   120
CAAGGGATTG GAATTGAGGA AGACTGTTAC TACAGTTGAG ACTCAGAACT TGGAAGGCCT   180
GCATCATGAT GGCCAATTCT GCCATAAGCC CTGTCCTCCA GATGTGAACA TGGAATCATC   240
AAGGAATGCA CACTCACCAG CAACACCAAG TGCAAAGAGG AAGGATCCAG ATCTAACTTG   300
GGGTGGCTTT GTCTTCTTCT TTTGCCAATT CCACTAATTG TTTGGGTGAA GAGAAAGGAA   360
GTACAGAAAA CATGCAGAAA GCACAGAAAG GAAAACCAAG GTTCTCATGA ATCTCCAACC   420
TTAAATCCTG AAACAGTGGC AATAAATTTA TCTGATGTTG ACTTGAGTAA ATATATCACC   480
ACTATTGCTG GAGTCATGAC ACTAAGTCAA GTTAAAGGCT TTGTTCGAAA GAATGGTGTC   540
AATGAAGCCA AAATAGATGA GATCAAGAAT GACAATGTCC AAGACACAGC AGAACAGAAA   600
GTTCAACTGC TTCGTAATTG GCATCAACTT CATGGAAAGA AAGAAGCGTA TGACACATTG   660
ATTAAAGATC TCAAAAAAGC CAATCTTTGT ACTCTTGCAG AGAAAATTCA GACTATCATC   720
```

CTCAAGGACA TTACTAGTGA CTCAGAAAAT TCAAACTTCA GAAATGAAAT CCAAAGCTTG 780

GTCTAGAGTG AAAAACAACA AATTCAGTTC TGAGTATATG CAATTAGTGT TTGAAAAGAT 840

TCTTAATAGC TGGCTGTAAA TACTGCTTGG TTTTTTACTG GGTACATTTT ATCATTTATT 900

AGCGCTGAAG AGCCAACATA 920

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 103 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala
1 5 10 15

Arg Leu Ser Ser Lys Ser Val Asn Ala Gln Val Thr Asp Ile Asn Ser
20 25 30

```
Lys Gly Leu Glu Leu Arg Lys Thr Val Thr Thr Val Glu Thr Gln Asn
          35                  40                  45

Leu Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro
    50                  55                  60

Pro Asp Val Asn Met Glu Ser Ser Arg Asn Ala His Ser Pro Ala Thr
65                  70                  75                  80

Pro Ser Ala Lys Arg Lys Asp Pro Asp Leu Thr Trp Gly Gly Phe Val
                85                  90                  95

Phe Phe Phe Cys Gln Phe His
            100
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 857 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
CACTTCGGAG GATTGCTCAA CAACCATGCT GGGCATCTGG ACCCTCCTAC CTCTGGTTCT    60
TACGTCTGTT GCTAGATTAT CGTCCAAAAG TGTTAATGCC CAAGTGACTG ACATCAACTC   120
CAAGGGATTG GAATTGAGGA AGACTGTTAC TACAGTTGAG ACTCAGAACT TGGAAGGCCT   180
GCATCATGAT GGCCAATTCT GCCATAAGCC CTGTCCTCCA GATGTGAACA TGGAATCATC   240
AAGGAATGCA CACTCACCAG CAACACCAAG TGCAAAGAGG AAGTGAAGAG AAAGGAAGTA   300
CAGAAAACAT GCAGAAAGCA CAGAAAGGAA AACCAAGGTT CTCATGAATC TCCAACCTTA   360
AATCCTGAAA CAGTGGCAAT AAATTTATCT GATGTTGACT TGAGTAAATA TATCACCACT   420
ATTGCTGGAG TCATGACACT AAGTCAAGTT AAAGGCTTTG TTCGAAAGAA TGGTGTCAAT   480
GAAGCCAAAA TAGATGAGAT CAAGAATGAC AATGTCCAAG ACACAGCAGA ACAGAAAGTT   540
CAACTGCTTC GTAATTGGCA TCAACTTCAT GGAAAGAAAG AAGCGTATGA CACATTGATT   600
AAAGATCTCA AAAAAGCCAA TCTTTGTACT CTTGCAGAGA AAATTCAGAC TATCATCCTC   660
```

```
AAGGACATTA CTAGTGACTC AGAAAATTCA AACTTCAGAA ATGAAATCCA AAGCTTGGTC      720

TAGAGTGAAA AACAACAAAT TCAGTTCTGA GTATATGCAA TTAGTGTTTG AAAAGATTCT      780

TAATAGCTGG CTGTAAATAC TGCTTGGTTT TTTACTGGGT ACATTTTATC ATTTATTAGC      840

GCTGAAGAGC CAACATA                                                     857
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 86 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala
1               5                   10                  15

Arg Leu Ser Ser Lys Ser Val Asn Ala Gln Val Thr Asp Ile Asn Ser
            20                  25                  30
```

Lys Gly Leu Glu Leu Arg Lys Thr Val Thr Thr Val Glu Thr Gln Asn
35 40 45

Leu Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro
50 55 60

Pro Asp Val Asn Met Glu Ser Ser Arg Asn Ala His Ser Pro Ala Thr
65 70 75 80

Pro Ser Ala Lys Arg Lys
85

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TATGTTGGCT CTTCAGCGCT A 21

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGTGTCATAC GCTTC 15

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGAATTCATA CCAAGTGCAG ATGGTA 26

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATCAAGCTTC ACCCAAACAA TTAGTGG 27

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 257 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AATTCATACC AAGTGCAGAT GTAAACCAAA CTTTTTTTGT AACTCTACTG TATGTGAACA 60

CTGTGACCCT TGCACCAAAT GTGAACATGG AATCATCAAG GAATGCACAC TCACCAGCAA 120

CACCAAGTGC AAAGAGGAAG GATCCAGATC TAACTTGGGG TGGCTTTGTC TTCTTCTTTT 180

GCCAATTCCA CTAATTGTTT GGGTGAAGCT TGGATCCGGA GAGCTCCCAA CGCGTTGGAT 240

GCATAGCTTG AGTATTC 257

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1126 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAGCGCTGAA GAGCCAACAT ATTTGTAGAT TTTTAATATC TCATGATTCT GCCTCCAAGG 60

ATGTTTAAAA TCTAGTTGGG AAAACAAACT TCATCAAGAG TAAATGCAGT GGCATGCTAA 120

GTACCCAAAT AGGAGTGTAT GCAGAGGATG AAAGATTAAG ATTATGCTCT GGCATCTAAC 180

ATATGATTCT GTAGTATGAA TGTAATCAGT GTATGTTAGT ACAAATGTCT ATCCACAGGC 240

TAACCCCACT CTATGAATCA ATAGAAGAAG CTATGACCTT TTGCTGAAAT ATCAGTTACT 300

```
GAACAGGCAG GCCACTTTGC CTCTAAATTA CCTCTGATAA TTCTAGAGAT TTTACCATAT   360
TTCTAAACTT TGTTTATAAC TCTGAGAAGA TCATATTTAT GTAAAGTATA TGTATTTGAG   420
TGCAGAATTT AAATAAGGCT CTACCTCAAA GACCTTTGCA CAGTTTATTG GTGTCATATT   480
ATACAATATT TCAATTGTGA ATTCACATAG AAAACATTAA ATTATAATGT TTGACTATTA   540
TATATGTGTA TGCATTTTAC TGGCTCAAAA CTACCTACTT CTTTCTCAGG CATCAAAAGC   600
ATTTTGAGCA GGAGAGTATT ACTAGAGCTT TGCCACCTCT CCATTTTTGC CTTGGTGCTC   660
ATCTTAATGG CCTAATGCAC CCCCAAACAT GGAAATATCA CCAAAAAATA CTTAATAGTC   720
CACCAAAAGG CAAGACTGCC CTTAGAAATT CTAGCCTGGT TTGGAGATAC TAACTGCTCT   780
CAGAGAAAGT AGCTTTGTGA CATGTCATGA ACCCATGTTT GCAATCAAAG ATGATAAAAT   840
AGATTCTTAT TTTTCCCCCA CCCCCGAAAA TGTTCAATAA TGTCCCATGT AAAACCTGCT   900
ACAAATGGCA GCTTATACAT AGCAATGGTA AAATCATCAT CTGGATTTAG GAATTGCTCT   960
TGTCATACCC TCAAGTTTCT AAGATTTAAG ATTCTCCTTA CTACTATCCT ACGTTTAAAT  1020
```

ATCTTTGAAA GTTTGTATTA AATGTGAATT TTAAGAAATA ATATTTATAT TTCTGTAAAT 1080

GTAAACTGTG AAGATAGTTA TAAACTGAAG CAGATACCTG GAACCA 1126

WHAT IS CLAIMED IS:

1. A composition comprising an isolated Fas antigen polypeptide that has a portion of the Fas antigen intracellular region, a portion of the Fas antigen extracellular region, and wherein at least a portion of the Fas antigen transmembrane region is deleted.

2. The composition of claim 1, wherein a significant portion of the Fas antigen transmembrane region is deleted from the Fas antigen polypeptide.

3. The composition of claim 1, wherein the Fas antigen polypeptide is a human Fas antigen polypeptide.

4. The composition of claim 3, wherein the Fas antigen polypeptide includes a 10 residue long contiguous sequence as set forth by the contiguous amino acid sequence Lys Cys Lys Glu Glu Val Lys Arg Lys Glu (position 164 to 173 of SEQ ID NO:4).

5. The composition of claim 4, wherein the Fas antigen polypeptide includes about a 20 residue long contiguous sequence from SEQ ID NO:4.

6. The composition of claim 5, wherein the Fas antigen polypeptide includes about a 50 residue long contiguous sequence from SEQ ID NO:4.

7. The composition of claim 6, wherein the Fas antigen polypeptide includes about a 100 residue long contiguous sequence from SEQ ID NO:4.

8. The composition of claim 7, wherein the Fas antigen polypeptide includes the contiguous amino acid of SEQ ID NO:4.

9. The composition of claim 1, wherein the Fas antigen polypeptide is a recombinant polypeptide.

10. The composition of claim 1, wherein the Fas antigen polypeptide is linked to a carrier protein.

11. The composition of claim 1, wherein the Fas antigen polypeptide is dispersed in a pharmacologically acceptable carrier.

12. A polypeptide comprising a sequence region that consists of at least a 10 residue long contiguous sequence as set forth by the contiguous amino acid sequence from Lys at position 164 to Glu at position 173 of SEQ ID NO:4.

13. The polypeptide of claim 12, wherein the sequence region consists of at least about a 25 residue long contiguous sequence from SEQ ID NO:4.

14. The polypeptide of claim 13, wherein the sequence region consists of at least about a 60 residue long contiguous sequence from SEQ ID NO:4.

15. The polypeptide of claim 14, wherein the sequence region consists of at least about a 120 residue long contiguous sequence from SEQ ID NO:4.

16. The polypeptide of claim 15, wherein the sequence region consists of at least about a 200 residue long contiguous sequence from SEQ ID NO:4.

17. The polypeptide of claim 16, wherein the sequence region consists of at least about a 298 residue long contiguous sequence from SEQ ID NO:4.

18. The polypeptide of claim 17, wherein the sequence region consists of the 314 residue long contiguous amino acid sequence of SEQ ID NO:4.

19. The polypeptide of claim 12, wherein the polypeptide is about 500 amino acid residues long.

20. The polypeptide of claim 19, wherein the polypeptide is about 250 amino acid residues long.

21. The polypeptide of claim 20, wherein the polypeptide is about 100 amino acid residues long.

22. The polypeptide of claim 21, wherein the polypeptide is about 50 amino acid residues long.

23. The polypeptide of claim 22, wherein the polypeptide is about 20 amino acid residues long.

24. An antibody that binds to a Fas antigen epitope that includes a portion of the Fas antigen intracellular region, a portion of the Fas antigen extracellular region and that does not include a significant portion of the Fas antigen transmembrane region.

25. The antibody of claim 24, wherein the antibody is a monoclonal antibody.

26. A nucleic acid segment comprising a soluble Fas antigen coding region that encodes a Fas antigen polypeptide that has a portion of the Fas antigen intracellular region, a portion of the Fas antigen extracellular region, and that lacks a significant portion of the Fas antigen transmembrane region.

27. The nucleic acid segment of claim 26, wherein the encoded Fas antigen polypeptide is a human Fas antigen polypeptide.

28. The nucleic acid segment of claim 27, wherein the encoded Fas antigen polypeptide includes a 10 residue long contiguous amino acid sequence as set forth by the

contiguous amino acid sequence from Lys at position 164 to Glu at position 173 of SEQ ID NO:4.

29. The nucleic acid segment of claim 28, wherein the nucleic acid segment includes a contiguous nucleic acid sequence as set forth by the contiguous nucleic acid sequence from position 515 to position 544 of SEQ ID NO:3.

30. The nucleic acid segment of claim 28, wherein the encoded Fas antigen polypeptide includes a 50 residue long contiguous amino acid sequence from SEQ ID NO:4.

31. The nucleic acid segment of claim 30, wherein the nucleic acid segment includes a 150 nucleotide long contiguous nucleic acid sequence from SEQ ID NO:3.

32. The nucleic acid segment of claim 30, wherein the encoded Fas antigen polypeptide includes a 298 residue long contiguous amino acid sequence from SEQ ID NO:4.

33. The nucleic acid segment of claim 32, wherein the nucleic acid segment includes the 1336 nucleotide long contiguous nucleic acid sequence of SEQ ID NO:3.

34. The nucleic acid segment of claim 26, wherein the Fas antigen coding region is positioned under the control of a promoter that directs its expression.

35. The nucleic acid segment of claim 34, wherein the Fas antigen coding region is positioned under the control of a recombinant promoter.

36. The nucleic acid segment of claim 34, wherein the Fas antigen coding region is positioned, in reverse orientation, under the control of a promoter that directs the expression of an antisense nucleic acid segment.

37. The nucleic acid segment of claim 26, further defined as a recombinant vector.

38. A recombinant host cell incorporating a nucleic acid segment that comprises an isolated Fas antigen coding region encoding a Fas antigen polypeptide that has a portion of the Fas antigen intracellular region, a portion of the Fas antigen extracellular region, and that lacks a significant portion of the Fas antigen transmembrane region.

39. The recombinant host cell of claim 38, further defined as a eukaryotic host cell.

40. The recombinant host cell of claim 38, wherein the nucleic acid segment is introduced into the cell by means of a recombinant vector.

41. The recombinant host cell of claim 40, wherein the host cell expresses the nucleic acid segment to produce the encoded Fas antigen polypeptide.

42. The recombinant host cell of claim 41, wherein the host cell secretes the encoded Fas antigen polypeptide.

43. A method of using a DNA segment that encodes a soluble Fas antigen polypeptide, comprising the steps of:

(a) preparing a recombinant vector in which a coding region encoding a Fas antigen that lacks a significant portion of the transmembrane region is positioned under the control of a promoter;

(b) introducing said recombinant vector into a recombinant host cell;

(c) culturing the recombinant host cell under conditions effective to allow expression of the soluble Fas antigen polypeptide; and

(d) collecting said expressed soluble Fas antigen polypeptide.

44. A recombinant Fas antigen polypeptide that lacks a significant portion of the transmembrane region, prepared by expressing a Fas antigen polypeptide in a recombinant host cell and purifying the expressed Fas antigen polypeptide away from total recombinant host cell components.

45. A method for detecting a cell that produces a soluble Fas antigen, comprising obtaining nucleic acids from a cell and identifying a nucleic acid segment that

encodes a Fas antigen that lacks a significant portion of the Fas antigen transmembrane region.

46. The method of claim 45, wherein said nucleic acid segment is identified by length.

47. The method of claim 45, wherein said nucleic acid segment is identified by sequence.

48. A method for detecting a soluble Fas antigen polypeptide in a sample, comprising the steps of:

(a) obtaining a sample suspected of containing a soluble Fas antigen polypeptide that lacks a significant portion of the Fas antigen transmembrane region;

(b) contacting said sample with a first antibody that binds to said soluble Fas antigen polypeptide, under conditions effective to allow the formation of immune complexes; and

(c) detecting the immune complexes so formed.

49. The method of claim 48, wherein said immune complexes are detected by means of a second antibody having binding affinity for said soluble Fas antigen polypeptide and a third antibody linked to a detectable label, the third antibody having binding affinity for said second antibody.

50. An immunodetection kit for detecting a soluble Fas antigen polypeptide that lacks a significant portion of the Fas antigen transmembrane region, the kit comprising:

(a) a first antibody that binds said soluble Fas antigen polypeptide, the first antibody being bound to a solid support;

(b) a suitably aliquoted composition of said soluble Fas antigen polypeptide;

(c) a second antibody that binds said soluble Fas antigen polypeptide; and

(d) a third antibody linked to a detectable label, the third antibody having binding affinity for said second antibody.

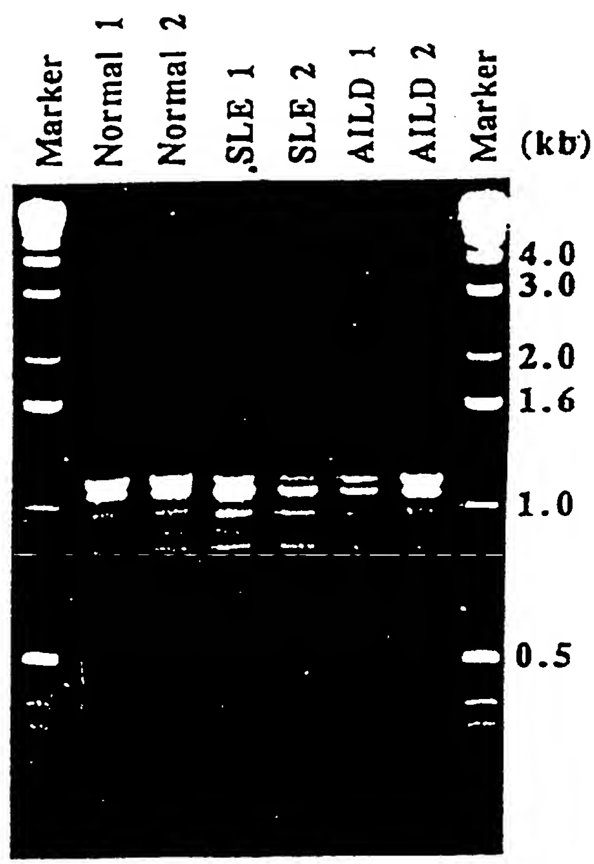
51. A method for diagnosing a Fas-associated disease, comprising determining the amount of a soluble Fas antigen that lacks a significant portion of the transmembrane region present within a biological sample from a patient, wherein an increased amount of said soluble Fas antigen, in comparison to the amount within a sample from a normal subject, is indicative of a patient with a Fas-associated disease.

52. The method of claim 53, wherein the amount of said soluble Fas antigen is determined by means of a molecular biological assay to determine the amount of a nucleic acid that encodes a soluble Fas antigen polypeptide.

53. The method of claim 53, wherein the amount of said soluble Fas antigen is determined by means of an immunoassay to determine the amount of a soluble Fas antigen polypeptide.

54. The method of claim 53, wherein said biological sample is a blood sample.

55. The method of claim 53, further defined as a method for diagnosing systemic lupus erythematosus (SLE) or angioimmunoblastic lymphadenopathy (AILD).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Normal 2
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AILD 2
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1.0
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FIG. 1

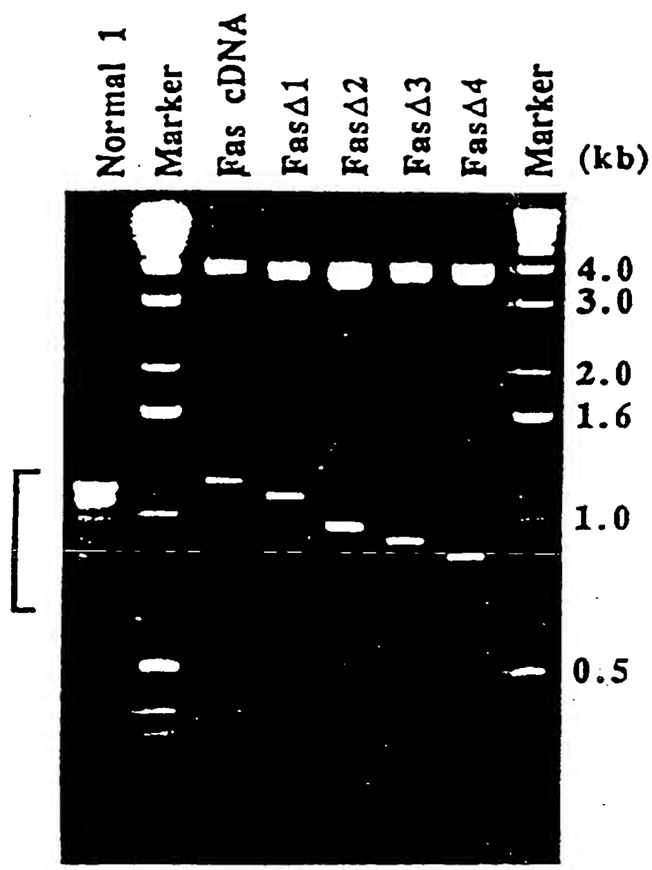
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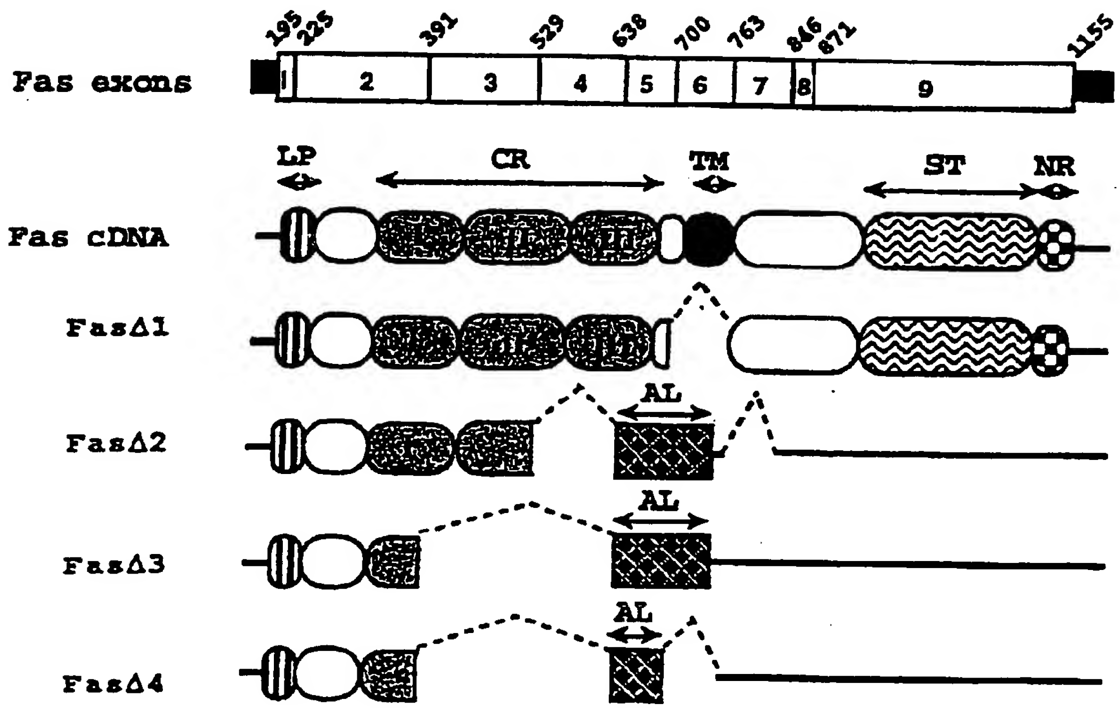
FIG. 2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763
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871
1155
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1
2
3
4
5
6
7
8
9
LP
CR
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ST
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FasΔ2
FasΔ3
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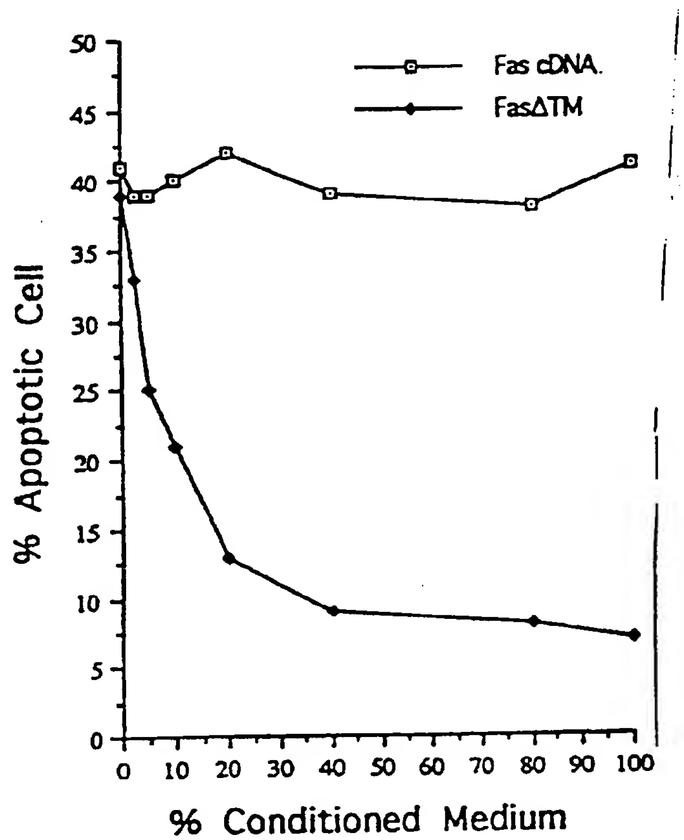
AL
AL
AL

FIG. 3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FasΔTM
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40
50
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90
100
% Conditioned Medium

FIG. 4

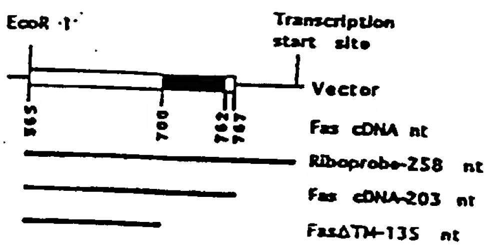
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FIG. 5

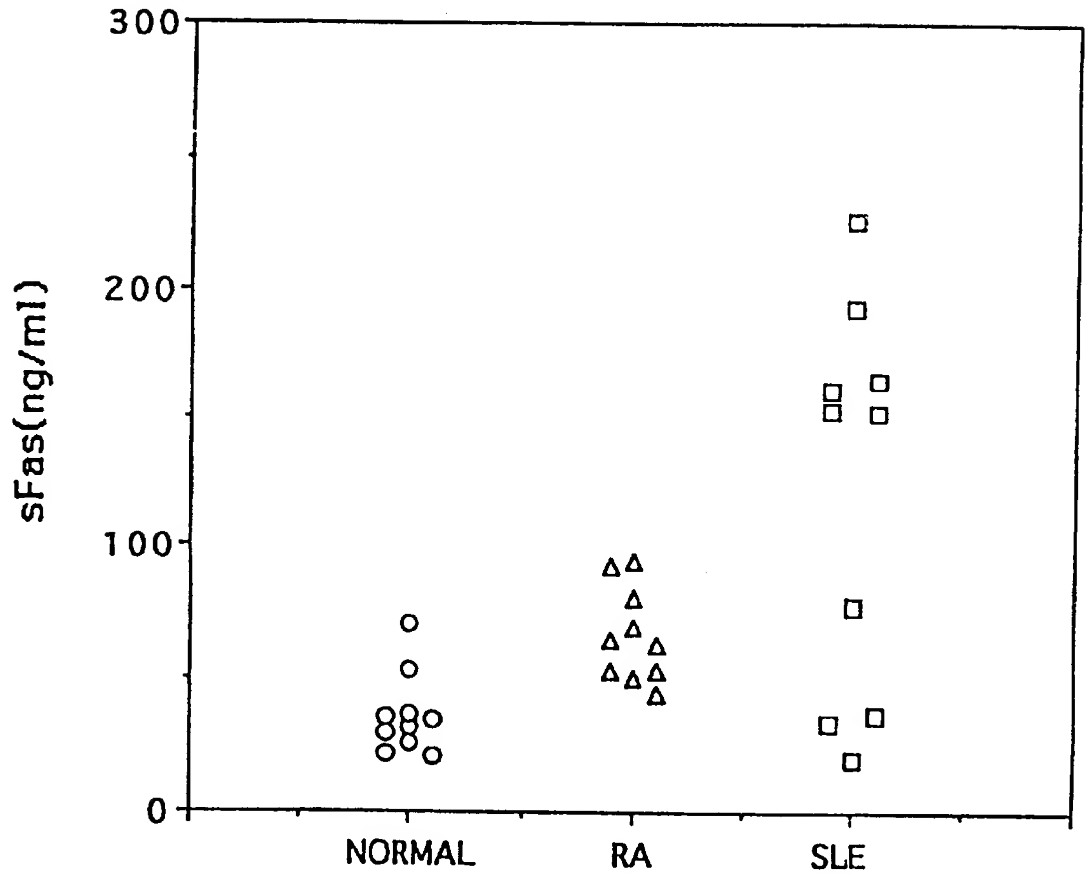

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/17083

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :536/23.1; 435/5, 7.1; 530/350, 351, 395; 424/85.1, 185.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 435/5, 7.1; 530/350, 351, 395; 424/85.1, 185.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Automated patent system (APS) and DIALOG (FILE=BIOCHEM). Key words: Fas, alternat?(w) splic?, trunc?, soluble, apotosis.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, A, 0 510 691 A1 (OSAKA BIOSCIENCE INSTITUTE) 28 October 1992, see entire document.	1-55
Y	Proc. Natl Acad Sci. (USA), Volume 90, published March 1993, Adachi et al., "Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice", pages 1756-1760, see entire document.	1-55
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 268, No. 15, published 25 may 1993, Itoh et al., "A Novel Protein Domain Required for Apoptosis", pages 10932-10937, see entire document.	1-55

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 MARCH 1996	18 APR 1996

Name and mailing address of the ISA/US	Authorized officer
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Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 66, published 26 July 1991, Itoh et al., "The Polypeptide Encoded by the cDNA for Human Cell Surface Antigen Fas Can Mediate Apoptosis", pages 233-243, see entire document.	1-55
Y	THE JOURNAL OF BIOLOGICAL CHEMSTRY, Volume 267, No. 15, published 25 May 1992, Oehm et al., "Purification and Molecular Cloning of the APO-1 Cell Surface Antigen, a Member of the Tumor Necrosis Factor/Nerve Growth Factor Receptor Superfamily", pages 10709-10715, see entire document.	1-55

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/17083

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/04; C12Q 1/68; G01N 33/53; C07K 14/475, 14/52; A61K 38/18, 38/19.